Vol. 43, No. 5 September, 1956

THE JOURNAL OF BIOCHEMISTRY

EDITED FOR THE JAPANESE BIOCHEMICAL SOCIETY

PUBLISHED BIMONTHLY

BY

THE JAPANESE BIOCHEMICAL SOCIETY

Tokyo University, Tokyo

AGENCIES

Charles E. Tuttle
Rutlant, Vt.
U.S.A.

Maruzen Co. Ltd.
Tokyo,
Japan

Santo Vanasio
Köln
Germany

PRINTED IN JAPAN

THE JOURNAL OF BIOCHEMISTRY was founded in 1922 by Prof. emeritus Dr. S. Kakiuchi and had been edited by him to volume 36 of the year of 1944. After the discontinuation during the War, this JOURNAL has been restarted by The Japanese Biochemical Society, following the serial number from volume 37 of the year of 1950.

This JOURNAL is collecting the original investigations on the subject in this country. It is published bimonthly, one volume per year.

Note to Contributors

All manuscripts should be carefully typewritten, triple-spaced, in English, French or German, and addressed to *The Editor, The Journal of Biochemistry, The Japanese Biochemical Society, c/o Department of Biochemistry, Faculty of Medicine, Tokyo University, Bunkyo-ku, Tokyo, Japan.*

Authors receive 50 reprints of their papers as a rule at cost; additional copies can be prepared by the order on cost accounts. All orders for reprints should be clearly noted on the article paper or in a sheet of paper attached to it. On submitting the paper, it is preferable to attach the non-traced charts besides original drawings, for circulation to Editors.

On request from authors to contribute to JOURNAL, the *Directions for preparation of manuscripts*, including styles of abbreviations of symbols, terms, units and periodicals, will be sent immediately.

Subscription

Subscription Price. ¥3,600 (U. S. $10.00) per volume, postage inclusive. Back issues are available for sale, from volume 1 up-to-date at ¥3,600 (U.S. $10.00) per volume, postage inclusive.

Remittances should be made preferably by check or draft payable to *The Japanese Biochemical Society, c/o Department of Biochemistry, Faculty of Medicine, Tokyo University, Bunkyo-ku, Tokyo, Japan.*

Foreign subscription is treated by the agencies of *Charles E. Tuttle, Co.*, Rutland, Vermont, U.S.A., *Santo Vanasio*, Köln, Germany, and *Maruzen Co. Ltd.*, Chuo-ku, Tokyo, Japan.

The Journal of Biochemistry, Vol. 43, No. 5, 1956

RESPIRATORY INCREASE AND PHOSPHORUS AND NITROGEN METABOLISM IN SWEET POTATO INFECTED WITH CERATOSTOMELLA FIMBRIATA*

By TAKASHI AKAZAWA and IKUZO URITANI

(From the Laboratory of Biochemistry, Faculty of Agriculture, Nagoya University, Anzyo)

(Received for publication, April 26, 1956)

Many intreresting metabolic changes occur in higher plant tissues when they are infected with pathogens. Among them an increase in respiratory rate is one of the most characteristic phenomena, and several suggestions have been put forward to explain this effect. P. J. Allen (*1*) studied it from the standpoint of phosphate metabolism and observed that in wheat infected with *Erysiphe gramnis* there occurred an increase in inorganic phosphate (P_i). He inferred that the respiratory increase may be due to the toxins of the pathogen or the substances produced** in the infected host which might act as uncouplers and accelerate the release of P_i and the accompanied regeneration of adenosine diphosphate (ADP), thus resulting in an increased level of the phosphate acceptor.*** From the recent studies of Lardy and Wellman (*2*), Siekevitz and Potter (*3*) and Slater (*4*), it is clear that the tissue respiration may be regulated by the reaction ATP→ADP+Pi, and Allen's research along these lines seems to be very meaningful.

During studies on the mechanism of respiratory increase of sweet potato tissue infected with *Ceratostomella fimbriata* (*C.f.*), we reported that uncouplers such as ipomeamaron accumulated in the infected part of this plant and stimulated the respiratory rate of the sound tissue (*5*). However, we have obtained evidence that the natural uncouplers exert potent inhibitory action on the pathogen itself (*6*, *7*), and may in fact play a part in the resisting action of the host plant. Therefore, we deemed it necessary to investigate the respiratory increase in relation to the question whether the uncoupling effect above-mentioned is

* Part 20 of Phytopathological Chemistry of Black Rot Sweet Potato.

** Personal communication.

*** Allen's interpretation was developed by A. Millerd and K. Scott recently (*Australian J Biol. Sci.*, **9**, 37 (1955)).

auxiliary in its nature, and whether there might be any other explanation for the stimulation observed.

In an attempt to answer these questions, a series of experiments has been carried out in which changes in phosphorus and nitrogen compounds were measured together with the respiration and its response to 2,4-dinitrophenol (DNP).

EXPERIMENTAL

Sweet potato (variety, Norin No. 1) was sliced into several pieces of 2–3 mm. thick, and a spore suspension of *C. f.* was sown on the surface of these slices, which were then incubated at 25°.

Uninoculated slices were served as control test for the effect of slicing under the same conditions. Under the present conditions, the fungus grew rapidly and, after about 48 hours, it spread over the surface and also penetrated into the sweet potato tissue; thereafter the growth declined gradually. In the present study, slices (2–3 mm. thick) from non-infected region (as determined microscopically) next to the infected (0.2–0.5 mm. thick) was taken to be assayed for various compounds concerned.

Respiratory Experiment

Small slices were taken from sound tissue, and the oxygen uptake was determined by the manometric technique. Experimental methods are shown in the legend of Table I. The DNP effect was determined by the respiratory increase caused by its addition in a final concentration of 5×10^{-5} *M*. DNP was most effective at this concentration both in the infected sweet potato and in the control.

Each value represents the average of duplicate determinations.

Fractionation

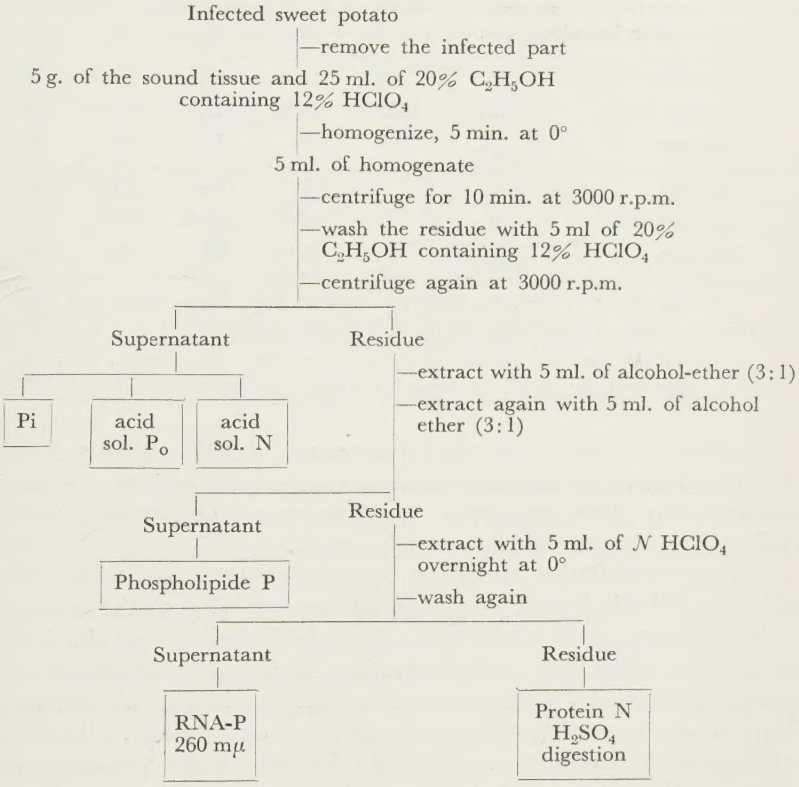
The method of Ogur and Rosen (*8*), was employed with slight modifications as shown schematically in Diagram 1. Phosphate was determined by Nakamura's method (*9*), and nitorogen by the micro-Kejldahl method. Ribonucleic acid (RNA) was estimated by absorption at 260 mμ by Shimadzu quartz spectro-photometer. Protein nitrogen was analysed by Howe's method (*10*).

RESULTS

1. Changes in the rate of respiration after inoculation with *C.f.*, and the enhancement by DNP are shown in Table I. In contrast to the nearly constant rate of respiration of the control with and without addition of DNP, the increase in respiration of the infected sweet potato reached a maximum at 48–72 hours, and decreased thereafter. The appropriate increase (80 per cent) caused by DNP dropped to about 30 per cent in the period of 48–72 hours, but recovered to the same level as

DIAGRAM 1

Fractionation Method for Phosphorus and Nitrogen Compounds in the Infected Sweet Potato



that of the control at the later stage (168 hours). Since the respiratory increase brought about by DNP is dependent on the acceleration of the reaction ATP→ADP+P_i in such a tissue, the values for the effect may indicate the speed of the above reaction in the tissues. It appears that the smaller DNP effect in the infected sweet potato may be caused by the acceleration of the reaction ATP→ADP+P_i in such tissue. This might occur by the promotion of breakdown of ATP and/or the utilization of ATP by some synthetic reaction. Indeed, from our data on the increase in the activity of respiratory enzymes such as cytochrome oxidase and polyphenol oxidase in the infected sweet potato tissue (*11*),

TABLE I

The Respiration of Infected and Sound Sweet Potato and the DNP Effect

Time after infection	*hrs.*	0	24	48	72	168
Control	*µl.*	77.3	88.0	80.5	77.6	93.7
Control DNP	*µl.*	138.7	156.0	143.0	149.0	178.0
Increase	*%*	80	77	78	92	90
Infected	*µl.*		132.7	147.0	177.0	120.0
Infected DNP	*µl.*		208.0	186.0	232.0	231.0
Increase	*%*		56	26	31	93

Twenty slices (7 mm. in diameter and 0.5 mm. thick) were put into Warburg vessels containing 85 µM of phosphate buffer (pH 5.5) and 162 µM of sucrose in total volume of 2 ml. Gas phase, air; temp., 30°; 20 min. equilibration; DNP was added in a final concentration of 5×10^{-5} *M*.

it is natural to suppose that the enhancement of ATP-utilizing reactions such as synthesis of enzyme proteins causes a concomitant respiratory increase. To clarify this point, the tissues were submitted to fractional analyses for several phosphorus compounds.

2. Representative data for phosphorus compounds are shown in Fig. 1. While both P_i and orgaic phosphate (P_o) of the control remained nearly constant with time, P_i in the sound part of the infected sweet potato decreased at the beginning, increased in the second stage, and dropped again, with concomitant increase in P_o. A constant P_i level in the secondary stage was observed occasionally. There was no significant difference between the infected sweet potato and the control tissue in their alcohol-ether soluble fraction containing phospholipide and in the cold $HClO_4$-extractable fraction containing RNA (Table II). The P_o formation in the sound tissue next to the infected tissue may thus indicate the activation of a phosphate cycle by which the energy of ATP is utilized in some way, resulting in the increase of the rate of ADP generation. But the observed increase in P_i at 24–48 hours and the minor effect of DNP on respiration may show the partial participation of ipomeamaron in the uncoupling effect or/and activation of adenosine triphosphatase (ATP-ase) which may be related to protoplasmic streaming and changes in permeability.

3. From the quantitative analysis of the phosphates, it was

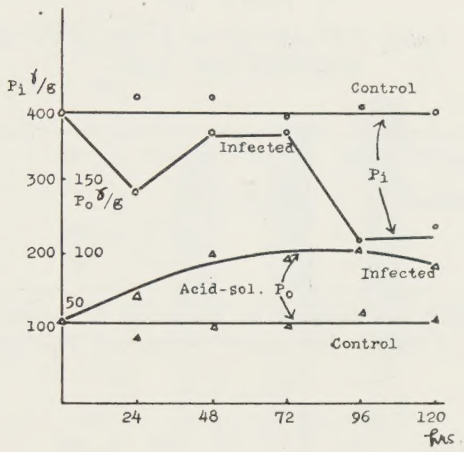


FIG. 1. Phosphate compounds level in the infected sweet potato and the sound one.

presumed that the anabolism of sweet potato tissue might be enhanced when it was infected with the pathogen. To elucidate this phenomenon more thoroughly, our attention was directed to the nitrogen metabolism, in particular, to the pattern of protein synthesis in the tissue. As shown in Fig. 2, the decrease in nitrogen compounds in the acid soluble fraction (amino acids and amides) is compensated by the concomitant increase in acid insoluble nitrogen compounds (proteins). The observed increase in proteins is one of the most typical anabolic phenomena of the infected plant. In Table III are shown the results of analyses for protein by Howe's method which has been applied to the analysis of serum protein. These data are also in accord with the above results.

DISCUSSION

French and Beevers have suggested that the increased respiration brought about when auxins are added to plant tissue may be due to the activation by the hormone of a system utilizing ATP (*12*). The DNP effect on the respiration of such an auxin-treated plant tissue is known to be less than that of the control (*13*). It has been shown by Pearson and Robertson (*14*) that the climacteric rise of apple in ripening process is due to the synthesis of respiratory enzyme proteins leading to the useful breakdown of ATP. However, Millerd *et al.*

TABLE II

Contents of Phospholipide and RNA in Infected and Sound Sweet Potato Tissue

Phospholipid-P

Time after infection, *hrs.*	0	24	48	72	96
Fresh sweet potato	95				
Control		94	100	94	95
Infected		99	98	90	98

Phospholipide-P was analyzed by estimation of inorganic phosphate after digestion of alcohol-ether soluble fraction with $HClO_4$.*

RNA-P

Time after infection, *hrs.*	0	24	48	72	96
Fresh sweet potato					
Control		48.8	50.0	48.7	42.5
Infected		53.0	51.5	47.4	44.6

RNA-P was estimated by the ultraviolet absorption at 260 mμ of cold N-$HClO_4$ extracted fraction*.

* Expressed as γ/g. fresh tissue.

(*15*) explained the mechanism of climacteric rise of avocado ripening as the acceleration of breakdown of ATP by ATP-ase or by some uncouplers. Our results represent an additional example of a similar regulatory mechanism in plant respiration. Synthetic events in the host are apparently stimulated by the infection of pathogens, and the resulting more rapid turnover of ATP is thought to bring about the respiratory increase observed. The uncoupling effect of metabolites such as ipomeamaron seems to be of relatively minor importance.

There is an increasing accumulation of many metabolites in the sound tissue adjacent to the infected tissue in parallel with the increase in respiration, and most of them may be concerned in some way with the resistance of the sweet potato to the pathogens (*16*). Besides metabolites of lower molecular weight thermolabile and non-dialyzable fraction having resisting action is produced in the sound part of the infected sweet potato. Possibly the resisting components may be the specific proteins such as the antiinflammatory factor of Menkin (*17*),

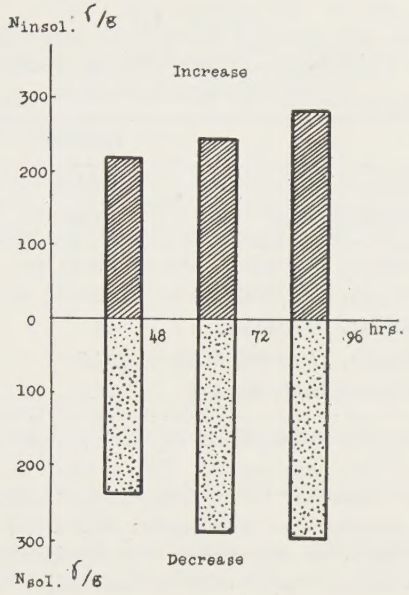


FIG. 2. Change of the amounts of nitrogen fraction in the infected sweet potato.

$N_{sol.}$ in the figure is expressed as the difference of the acid-soluble nitrogen fraction in the control from that in the infected sweet potato, and $N_{insol.}$ as the difference of acid-insoluble nitrogen fraction in the infected sweet potato from that in the control. The values of the control were scarecely changed throughout the experimental stage.

properdin system, a facter of natural immunity (*18*), or certain enzymes which may damage the pathogen or break down the poisonous metabolites of it. As the another way in which the protein may participate in the resistance, the following assumption is possible. The protein in the sound part adjuscent to the infected is unstable as well as active; when the cells of the sound part are affected by the pathogen, the protein may be denatured rapidly, interact with the accumulated polyphenols, thus form the barrier against penetration of *C.f.* The rise in anabolic process observed in infected sweet potato is the unique phenomenon of infected plant tissues, and it is important to discuss this finding in relation to host resistance. Tomiyama *et al.* (*19*) reported the similar phenomenon in the white potato infected with *Phytophthora*

TABLE III

The Distribution of Nitrogen-Containing Substances in Infected and Sound Sweet Potato

		Infected	Control
Tissue	Water	69.4 %	67.2 %
	Crude protein	1.04	1.06
Juice	Total	989 *mg.*	1046 *mg.*
	Na_2SO_4 14 g./10 ml.	742	568
	Na_2SO_4 18 g./10 ml.	19	32.2
	Na_2SO_4 22 g./10 ml.	74.5	10.0
	Unprecipitable fraction	140.6	360.9

Three days after the inoculation of *C.f.*, the samples were prepared in the way shown in the Experimental. The amounts of crude protein in the samples were estimated by micro-Kjeldahl method, after H_2SO_4 digestion. The samples were homogenized with equal amounts of cold water and centrifuged, the supernatants were fractionated by the method of Howe. Each fraction was estimated by micro-Kjeldahl method, and the N amount was multiplied by 6.25, and expressed as the amount per 100 g. of tissue (wet weight).

infestans and presented an interpretation like ours.

SUMMARY

1. Changes in the metabolism of sweet potato tissue induced by infection with *Ceratostomella fimbriata* were studied.

2. The respiration of sound tissue adjacent to the infected tissue was about twice greater as much as the control 72 hours after the fungus inoculation; thereafter it dropped. Rate of the respiratory increase due to DNP addition was reciprocal to that of the respiratory increase caused by the fungus infection, and the finding was assumed to be caused by the acceleration of ADP generation in the tissue.

3. In the sound tissue next to the infected, inorganic phosphate decreased in line with organic phosphate formation, and a concomitant increase of acid insoluble nitorogen conpounds (protein) occurred, a decrease of acid soluble nitrogen compounds (amino acids and amides) being also observed. These facts indicate an activation of anabolism

in the infected sweet potato, but occasionally at one stage (72 hours) Pi increase was found, and the partial participation of an uncoupling reaction or the activation of ATP-ase is suggested.

4. In the infected sweet potato, the levels of phospholipide and RNA remained unchanged.

We wish to express our thanks to profs. Y. Sumiki and S. Funahashi, University of Tokyo, for their cordial advices and helpful suggestions and also for the assistance of Mrs. M. Uritani, Miss Y. Hirata and Mr. M. Egawa.

This communication was already presented before the meeting of the Agriculturel Chemical Society of Japan (Tokyo, December 18, 1954).

REFERENCES

(*1*) Allen, P. J., *Phytopath.*, **43**, 22 (1953)
(*2*) Lardy, H. K., and Wellman, H., *J. Biol. Chem.*, **195**, 215 (1953); **201**, 357 (1953)
(*3*) Siekevitz, P., and Potter, van R., *J. Biol. Chem.*, **201**, 1 (1953)
(*4*) Slater, E. C., *Nature*, **172**, 975 (1953)
(*5*) Uritani, I., Akazawa, T., and Uritani, M., *Nature*, **174**, 1060 (1954)
(*6*) Utitani, I., and Akazawa, T., *Science*, **121**, 2137 (1954)
(*7*) Akazawa, T., and Uritani, I., *J. Agr. Chem. Soc. Japan*, **29**, 337 (1954)
(*8*) Ogur, M., and Rosen, G., *Arch. Biochem.*, **25**, 262 (1950)
(*9*) Nakamura, M., *J. Agr. Chem. Soc. Japan*, **24**, 1 (1950)
(*10*) Howe, P.E., *J. Biol, Chem.*, **49**, 109 (1921)
(*11*) Uritani, I., and Akazawa, T., *J. Agr. Chem. Soc. Japan*, **28**, 205 (1954)
(*12*) French, R. C., and Beevers, H., *Am. J. Bot.*, **40**, 660 (1953)
(*13*) Bonner, J., and Bandurski, R. S., *Ann. Rev. Plant Physiol.*, **3**, 59 (1953)
(*14*) Pearson, H. A., and Robertson, R. W., *Aust. J. Biol. Sci.*, **7**, 1 (1954)
(*15*) Millerd, A., Bonner, J., and Biale, J. B., *Plant Physiol.*, **28**, 521 (1953)
(*16*) Uritani, I., and Miyano. M., *Nature*, **175**, 812 (1955)
(*17*) Menkin, V., Newer concepts of inflammation, CHARLES C THOMAS PUBLISHER Springfield, Illinois, (1948)
(*18*) Pillemer, L., *Science*, **120**, 279 (1954), **122**, 545 (1955)
(*19*) Tomiyama, K., *Agr., Technique (Japan)*, **10**, No. 4 (1955)

The Journal of Biochemistry, Vol. 43, No. 5, 1956

METABOLIC ACTIVATION OF WHITE POTATO TISSUE INFECTED WITH CERATOSTOMELLA FIMBRIATA*

By TAKASHI AKAZAWA

(From the Laboratory of Biochemistry, Faculty of Agriculture, Nagoya University, Anzyo)

(Received for publication, April 26, 1956)

Previously we reported the activation of metabolism in sweet potato tissue infected with *Ceratostomella fimbriata* (*C. f.*) through the observation of respiratory increase, soluble organic phosphate accumulation and protein synthesis, and suggested the fact that the mechanism of respiratory increase in infected plant might be caused mainly by the activation of metabolism utilizing the ATP cycle in the tissue (*1*). The work was done with a variety of sweet potato, Norin No. 1. This variety, although fairly resistant to *C. f.*, decays after prolonged exposure to the infection. To clarify the relationship between metabolic activation in infected plant and their resistance, it seemed appropriate to study the metabolic alteration of such a tissue as of the white potato which is most resistant to this pathogen. Consequently the metabolic activity of white potato infected with *C. f.* was analyzed by the procedures similar to those employed in the study of infected sweet potato. Although there is no full agreement, similar overall phenomena were observed. These findings lead us to believe that the activation of metabolic functions in the indicated tissues is a general response when the pathogen infects the host, and reflects the active reaction of the host to the invading organisms.

One mutant of *C. f.* attacks one kind of the tropical plant, *Syngoinium auritum* (*2*). Although this mutant infects sweet potato only rarely, it attacks white potato more severely than the normal strain does and in white potato infected with *C. f.* from *Syngonium auritum*, respirotory increase is also observed.

Mechanism of the resistance of white potato against *Phytophtora infestans* has been studied in relation to the cellular physiology by K. Tomiyama *et al.* (*3*).

---

* Part 21 of Phytopathological Chemistry of Black Rot Sweet Potato.

EXPERIMENTAL

White potato tissue was subjected to fungus inoculation in a manner similar to that described for sweet potato (*1*), but the growth of the fungus was not so good as in sweet potato tissue. About 48 hours after the inoculation the surface of white potato became brown, but the penetration of the fungus was very slight and was subsequently greatly inhibited. Experimental methods for the measurements and analyses were the same as those described previously, unless otherwise mentioned. (*1*)

RESULTS

1. When white potato was infected with *C. f.*, a respiratory increase was observed, but the extent of this increase, as well as the degree of fungus penetration was not so large as compared with that observed in the sweet potato (Fig. 1). The respiratory increase was determined also in the white potato infected with *C. f.* from *Syngonium auritum* (Table I).

2. As shown in Fig. 2, inorganic phosphate (P_i) decreased in the infected white potato, and organic phosphate (P_o) simultaneously increased. The changes, however, were not so large as in sweet potato. Furthermore, no measurable change of RNA content could be found (Table II).

3. The results of nitrogen analyses also indicate an activation of metabolic activity in infected white potato. Representative results of the analyses of acid-soluble nitrogen compounds ($N_{sol.}$) and acid insoluble ones ($N_{insol.}$) are shown in Fig. 3. In this case, the utilization of $N_{sol.}$ is followed by the synthesis of $N_{insol.}$

DISCUSSION

The results show that sound white potato tissue adjacent to area infected with *C. f.* manifests a respiratory increase, and it is suggested that this is caused by the activation of an ATP-utilizing reaction. It should be stressed that these phenomena could be observed in the infected tissue of white potato which was most resistant to *C. f.*, as well as in those of sweet potato tissue which are less resistant. It is fruitful to consider the relationship between the degree of respiratory increase due to fungus infection and the degree of penetration of the fungus. In the case of sweet potato infected with *C. f.*, the respiratory amount, 15–24 hours after the fungus inoculation was about 1.5 times greater than the control. After 48–72 hours, however, respiration was raised

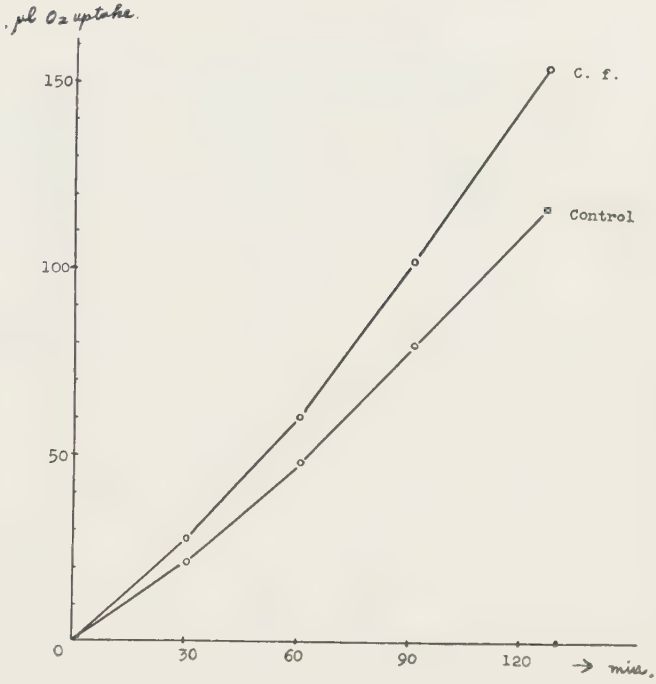


FIG. 1. Respiratory increase of white potato infected with *Ceratostomella fimbriata.*

Twenty slices (7 mm. in diameter and 0.5 mm. thick) were put into the Warburg vessels containing 85 μM of phosphate buffer (pH 5.5) and 162 μM of sucrose in a total volume of 2 ml. Gas phase, air; temp., 30°; 20 min. equilibration.

Sound part of 48 hrs.-infected white potato was subjected to the analysis.

up to 2 times with increasing penetration. *C. f.* from *Syngonium auritum* does not penetrate deeply into sweet potato tissue, and the concomitant respiratory increase is smaller. Thus the respiratory increase caused yb fungus penetration seems to be in parallel with the depth of penetration. However, there is an upper limit to the degree of respiratory increase. This limit is not exceeded even in severe fungus infection. Whereas *C. f.* penetrates the tissue of the white potato only slightly, the *C. f.* from *Syngonium auritum* is able to penetrate it readily and to bring about extensive damage. However, in both cases, the respiratory in-

TABLE I

Respiratory Increase of Infected Tissues

	Time After infection	Infected with *Ceratostomella fimbriata* from sweet potato*	Infected with *Ceratostomella fimbriata* from *Syngonium auritum*
	hrs.	%	%
White	24	20—40	0—10
potato	48	20—40	20—40
	72	20—40	20—40
Sweet	24	40—50	20—40
potato	48	80—100	10—30
	72	100—120	

Experimental methods were the same as those of Fig. 1.

* Respiratory increase of infected tissue compared with that of the sound tissue (% increase).

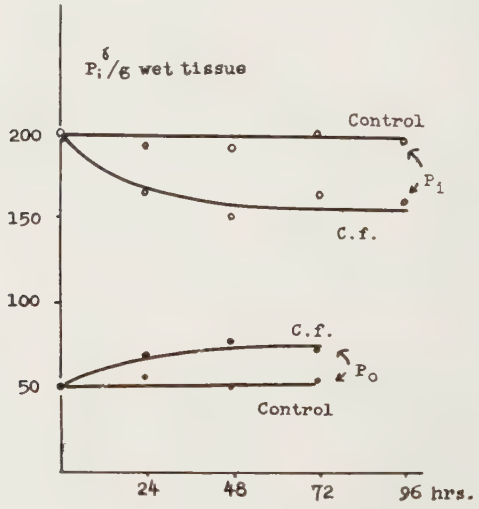


FIG. 2. Change of level in the phosphate compounds of infected potato.

Expressed value represents the change of phosphate compounds (γ/g. fresh weight).

TABLE II

RNA-P Content of White Potato Infected with Ceratostomella fimbriata

Time after inoculation	RNA-P of white potato (γ/g. fresh weight)		
hrs.	24	48	72
Control	24.4	21.5	24.4
C.f.	25.0	21.3	23.7

The RNA fraction was extracted with cold *N* $HClO_4$ and estimated by ultraviolet absorption at 260 mμ.

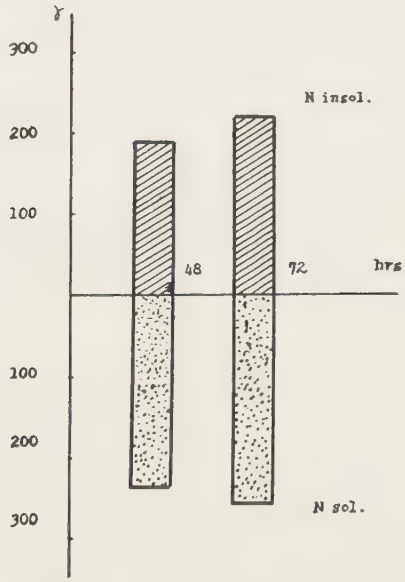


FIG. 3. Change of the amount of nitrogen compounds in white potato infected with *Ceratostomella fimbriata*.

The values were expressed in the same manner as shown in Part 20, Fig. 2.

crease 48 hours after the inoculation reaches about 1.4–1.5 times greater than the initial (Table I). In my opinion, the value may be the upper

TABLE III

Comparison of the Amount of Phosphorus and Nitrogen Compounds in Sweet Potato and White Potato
(P or N γ/g. fresh weight)

	P_i	P_o	RNA	Phospholipide	$N_{sol.}$	$N_{insol.}$
Sweet potato	400	50	50	90	700	1200
White potato	200	20–25	20		2000	1000

limit of respiration in the white potato tissue.

It can be seen that a decrease in P_i is accompanied by P_o increase, and the decrease in $N_{sol.}$ is in parallel with the increase in $N_{insol.}$ in white potato infected with *C. f.* The following difference between the fresh sweet potato and white potato should be noted: the former shows the higher content of P_i and the lower content of $N_{sol.}$ As there are no differences in the change of nitrogen levels due to fungus penetration between sweet potato and white potato, it seems plausible that P_i level is concerned with the capacity for respiratory increase (Table II).

The metabolic activation of infected plant is a response of the host plant to fungus penetration. However, the activation of anabolic events in the infected plant may in some way be related to a defense mechanism of host.

SUMMARY

1. Metabolic changes of white potato infected with *C. f.* to which it is strongly resistant were studied by methods similar to those used in a previous study of sweet potato.

2. In sound tissue next to infected tissue of white potato respiratory increase, P_i decrease and concomitant increase in P_o were observed. The degree of change in these factors was smaller than that observed in the sweet potato. But the increase in $N_{insol.}$ (protein) compensating the decrease in $N_{sol.}$ (amino acids and amides) reached the same level as in infected sweet potato. Thus the respiratory increase observed in white potato infected with *C. f.* is thought to be caused by the APT-utilizing reaction.

The author wishes to express his gratitude to Dr. I. Uritani, Nagoya Uni-

versity, for his sincere advice, and to Dr. L. Davis, University of Calif. at Los-Angels, for her gift of *Ceratostomella fimbriata* from *Syngonium auritum*.

This communication was presented before the meeting of the Biochemical Society of Japan (Kyoto, April 4, 1955).

REFERENCES

(*1*) Akazawa, T., and Uritani, I., *J. Biochem.*, **43**, 579 (1956)
(*2*) Davis, L. H., *Phytopath.*, **43**, 586 (1953)
(*3*) Tomiyama, K., *Agr. Tech.* (*Japan*), **10**, No. 4 (1955); *J. of Hokkaido Agr. Exptl. Station*, 24 (1954)

The Journal of Biochemistry, Vol. 43, No. 5, 1956

NATURE OF PROTEIN SYNTHESIS IN SWEET POTATO TISSUE INFECTED WITH CERATOSTOMELLA FIMBRIATA*

By TAKASHI AKAZAWA

(*From the Laboratory of Biochmestry, Faculty of Agriculture, Nagoya University, Anzyo*)

(Received for publication, April 26, 1956)

Studies on the Mechanism of respiratory increase in sweet potato infected with *Ceratostomella fimbriata* (*C. f.*) have indicated that this might be caused by the activation of a phosphate cycle linked to organic phosphate (P_o) accumulation and protein synthesis (*1*). Since similar phenomena were observed in infected white potato which is resistant to this pathogen (*2*), these findings may be of value in the elucidation of the resistance of the host plant and the pathogenicity of the parasite.

As already reported, respiratory enzymes such as cytochrome oxidase and polyphenol oxidase are activated in fungi-infected sweet potato (*3*), therefore it was thought that the protein synthesized during penetration of fungus might be composed of functional protein such as enzymes. To determine the nature of the protein synthesis the crude homogenate from the sound part adjacent to the infected part was fractionated into mitochondria, microsomes, and supernatant by centrifugation. An increase in protein nitrogen was found in each fraction. RNA is higher in the microsomes in parallel with the decrease in RNA in the supernatant. Since protein synthesis might be expected to be linked to an ATP system, mitochondrial activity of oxidative phosphorylation coupled to respiratory oxidation was also determined.

EXPERIMENTAL

Sweet potato (variety, Norin No. 1) was sliced into several pieces of 2–3 cm. thick, and a spore suspension of *C. f.* was distributed on the surface of these slices, which were then incubated at 25°. Uninoculated controls were allowed to stand at the same conditions to test the effect of slicing.

Fractionation Method—The sound part adjacent to the infected part of sweet potato

* Part 22 of Phytopathological Chemistry of Black Rot Sweet Potato.

was subjected to fractionation according to Schneider's method (*4*). The crude homogenate prepared in 0.25 *M* sucrose solution was centrifuged at $500\times g$ to remove large particles such as nuclei, starch and cell walls. The resulting supernatant was again centrifuged at $8000\times g$ for 10 minutes, and subsequently at $18000\times g$ for 60 minutes, to obtain mitochondria, microsomes, and supernatant. These were denoted as M_w, P_w and S_2, Each of them was subjected to analysis for RNA and protein as described in a previous paper (*1*).

Respiratory Oxidation and Oxidative Phosphorylation—The method for the preparation of mitochondria and the determination of oxidative phosphorylation activity were described in the previous paper (*5*). The details of the reaction are shown in each table.

The Determination of Adenosine Triphosphatase (ATP-ase) and of Phosphatase Activity—The same enzyme preparation as in oxidative phosphorylation was utilized. The initial and final phosphorus contents were analyzed by the method of Nakamura (*6*), after the addition of perchloric acid to precipitate protein.

RESULTS

As shown in Table I, protein nitrogen was higher in the infected sweet potato than in the control in every fraction (M_w, P_w and S_2). RNA is higher in the microsomes in parallel with a decrease in the supernatant, the total amount remaining constant.

TABLE I

The Amount of RNA and Protein in the Cytoplasmic Fractions of Both the Infected Sweet Potato and Sound One

		M_w	P_w	S_2	Total
Control	RNA-P	1.74	2.01	14.5	18.25
	Protein-N	66.7	47.3	309.0	423.0
Infected	RNA-P	1.84	3.54	13.0	18.38
	Protein-N	88.0	74.5	390.0	552.5

The estimated values were expressed as the γ/g. fresh tissue.

In order to determine the nature of functional protein synthesized, respiratory oxidation and oxidative phosphorylation activity of mitochondria were investigated. Both activities were higher in the infected sweet potato than in the control (Table II). However, oxidative phosphorylation activity was lower in both cases than that in the mitochondria of fresh sweet potato itself without slicing. This result might

TABLE II

Respiratory Oxidation and Oxidative Phosphorylation

Time after slicing *hrs.*		O_2*	P*	P/O	Protein N	O_2**
Fresh	Sweet potato	2.0	2.15	1.08		
48	Control	3.6	0.59	0.16	419	8.6
	Infected	6.7	3.6	0.54	577	11.6
72	Control	2.77	0	0	426	6.5
	Infected	7.61	3.2	0.42	595	12.8

* Uptake in μatoms/ml./hr.

** Uptake in μatoms/mg. N.

Reaction mixture contained 20 μM phosphate buffer (pH 7.2), 200 μM of sucrose, 20 μM of *a*-Ketoglutaric acid, 100 μM of glucose, 3 μM of ATP, 10 μM of $MgCl_2$ 10 μM of versene, 20 μM of NaF and 1 ml. of enzyme in a total volume of 2.3 ml. Incubation time, 60 min.

Incubation at 30° in Warburg vessels. The mitochonria were prepared according to the previous paper (*18*) using 45 g. of each tissue, and finally they were suspended in 3 ml. of the 0.5 *M* sucrose solution containing 0.02 *M* phosphate buffer and 0.2 *M* NaF.

depend on two factors. First there may be experimental difficulties in the preparation of the mitochondria. As already shown (*3*), the activity of polyphenol oxidase and the amount of polyphenol are enhanced in the sliced or the fungi-infected sweet potato, and during preparation mitochondria from such tissues are consequently exposed to a greater degree to the harmful effect of the resulting quinones. Indeed, we obtained evidence that the oxidative phosphorylation of sweet potato mitochondria was inhibited by the oxidation product of chlorogenic acid, a polyphenolic substance of sweet potato (*5*). Another factor may be an augumentation of ATP-ase activity in the sweet potato, since it can be shown that the ATP-ase activity is considerably increased in both the sliced and infected sweet potato tissues. However, addition of ethylenediamine tetraacetic acid (EDTA) and NaF to suppress the breakdown of ATP by this enzyme did not result in higher P/O ratio (Fig. 1), (Table II).

DISCUSSION

The correlation of RNA and protein synthesis in the microsomal

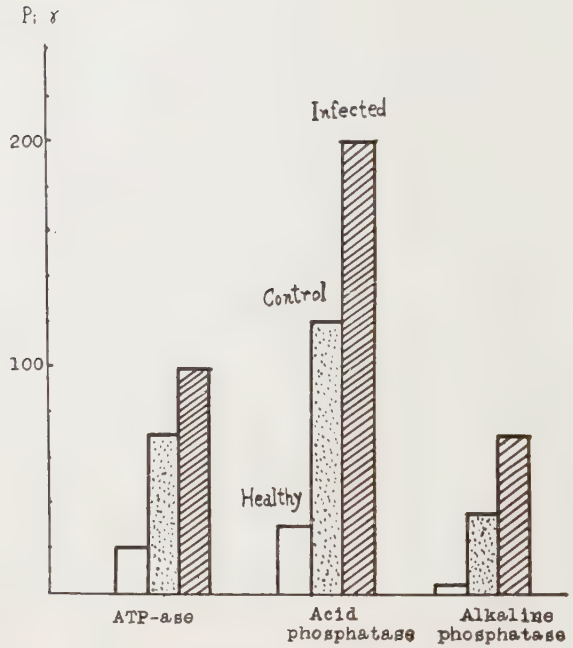


FIG. 1. Activities of ATP-ase and phosphatase (acid and alkaline) in the mitochondria of fresh cut down (Control) and infected sweet potato (Infected).

Enzymes were prepared by the same method in Table II except the omission of EDTA and NaF from the preparative medium of mitochondira, and suspended in the distilled water finally. Reaction was carried out 30°, for 40 min., then trichloroacetic acid was added to top the reaction and the split inorganic P_i was determined.

1. Reaction mixture for ATP-ase activity determination was as follows: 0.1 *M* succinate buffer (pH 4.7) 2 ml., 0.01 *M* ATP (Pabst. prepn.) solution 1 ml., the enzyme solution 1 ml.

2. Reaction mixture for acid phosphatase activity was as follows: 0.1 *M* succinate buffer (pH 4.0) 2 ml., 0.1 *M* Na β-glycerophosphate solution 1 ml., the enzyme solution 1 ml.

3. Reaction mixture for alkaline phosphatase activity was as follows: 0.1 *M* veronal buffer (pH 8.0) 2 ml., 0.1 *M* Na β-glycerophosphate solution 1 ml., the enzyme solution 1 ml.

fraction of infected sweet potato (shown by the data in Table I) agrees

with other studies of protein synthesis (*7*, *8*). During protein synthesis in the sound part next to the infected tissue, the RNA of the supernatant may be transferred into the microromes and changed to the microsomal where it may participate in the formation of all the cellular proteins. Oota and Osawa obtained some indications for the transfer of RNA in the seedling of *Vigna sesquipedalis* (*9*).

It should be stressed that in the sound part next to the infected part the activity of the enzymes bound to the mitochondria, such as the enzymes concerning respiratory oxidation and phosphorylation, increased concomitantly with the formation of mitochondria, and furthermore the respiratory activity per unit mitochondria weight was much higher than the control. This indicates a higher metabolic efficiency in the infected sweet potato. The respiratory increase in the infected sweet potato may be due to the acceleration of an ATP-utilizing reaction coupled to the synthesis of functional protein. The increment in metabolic activity due to the newly synthesized functional protein would be expected to lead to a more rapid rate of respiration.

The active state of the protoplasm of the infected sweet potato is not only a characteristic phenomenon, but also may be, in some way, concerned with the resistance of the host plant to the invading fungus. As already noticed, there may be several components in the synthesized proteins, which are resistant to the pathogen. We were able to observe that the particulate fraction in the sound part of sweet potato infected with *C. f.* considerably inhibited the germination of the fungus spore ; but it is unknoun whether or not this action plays a part in resistance *in vivo*. A similar opinion has been put forward by Walker *et al.* (*10*) in studies of tomato wilt. They found that the resistant tomato became susceptible when it was treated with DNP, KCN and other respiratory poisons, and suggested that the antibiotic may be a very labile fraction produced under coupling to the ATP-system. It would be of interest to elucidate the nature of the components which display the resistant function. As already described (*1*), the following assumption is possible as the another way in which the protein may participate in the resistance. The protein in the sound part next to the infected is unstable as well as active ; when the cells of the sound part are effected by the pathogen, the protein may be denatured rapidly and interacts with the accumulated polyphenols, thus forming the barrier against penetration of *C. f.* In this connection we have recently confirmed that the protein fraction of the sound part next to the infected part dif-

ferred electrophoretically from that of control. Further investigation are now in progress.

SUMMARY

1. To elucidate the nature of the anabolic processes of infected sweet potato, protein synthesis, and the activation of respiratory oxidation and oxidative phosphorylation were determined.

2. In the infected sweet potato, protein increased in every cellular fraction (microsomes, mitochondria, and supernatant). RNA was higher in the microsomal fraction in parallel with the decrease in supernatant. This protein synthesis may be carried out in the microsomes, which are rich in RNA.

3. Respiratory oxidation and the oxidative phosphorylation were higher in the mitochondira of infected sweet potato than in those of the control. The activity of respiratory oxidation per unit mitochondrial nitrogen was also higher than in the control.

4. ATP-ase and phosphatase activities were enhanced in the infected sweet potato and the physiological implication was discussed.

The author wishes to express his gratitude to Dr. I. Uritani, Nagoya University, for his sincere advice.

This communication was presented before the Meeting of the Agricultural Chemical Society of Japan (Tokyo, April 1, 1955).

REFERENCES

(*1*) Akazawa, T., and Uritani, I., *J. Biochem.*, **43**, 579 (1956)
(*2*) Akazawa, T., *J. Biochem.*, **43**, 589 (1956)
(*3*) Uritani, I., and Akazawa, T., *J. Agr. Chem. Soc. Japan*, **27**, 789 (1953)
(*4*) Schneider, W. C., and Hogeboom, G. H., *J. Biol. Chem.*, **183**, 123 (1950)
(*5*) Akazawa, T., and Uritani, I., *J. Biochem.*, **41**, 631 (1954)
(*6*) Nakamura, M., *J. Agr. Chem. Soc. Japan*, **41**, 631 (1954)
(*7*) Oota, Y., and Osawa, S., *Biochem. Biophys. Acta*, **15**, 163 (1954)
(*8*) Allfrey, V., Daly, M. M., and Mirsky, A. E., *J. Gen. Physiol.*, **37**, 157 (1953)
(*9*) Oota, Y., and Osawa, S., *Experientia*, X/**6**, 254 (1954)
(*10*) Walker, J. C., and Stahmann, M. A., *Ann. Rev. Plant Phyiol.*, **6**, 351 (1955)

The Journal of Biochemistry, Vol. 43, No. 5, 1956

STUDIES ON THE OXIDATIVE DECOMPOSITION OF UROCANIC ACID BY LIVER EXTRACT. I*

By KATASHI ICHIHARA, SHIGENOBU ITAGAKI, YOSHITOSHI SUZUKI, MAKIO UCHIDA and YUKIYA SAKAMOTO

(*From the Biochemical Department, Medical School, Osaka University, Osaka*)

(Received for publication, April 28, 1956)

About ten years ago Kumagai (*1*) of this laboratory conducted studies on the oxygen uptake in the decomposition of histidine and glutamic acid by the liver extract of the guinea pig, in which histidine deaminase and urocanicase were contained in a ratio of 1 : 2. He incubated the reacting solution at 37°, pH 8.5 for 20 hours using Warburg's apparatus and obtained the following data. This meant, that one

Substrate	O_2-consumption	Atom of oxygen consumed per mol. of substrate
L-Histidine (1.26 mg.)	−66.31 *μl.*	0.99 *atom*
L-Glutamic acid (0.876 mg.)	+ 2.00	0.00

atom of oxygen was consumed per molecule for the decomposition of urocanic acid, because histidine was deaminated to urocanic acid without oxygen uptake. From this fact, it was assumed at that time, that urocanic acid was converted to glutamic acid through hydantoinpropionic acid with the consumption of one atom of oxygen. However, all other investigaters claimed, that the pathway of urocanic acid to glutamic acid was hydrolytic. Moreover Kometani of this laboratory proved that hydantoinpropionic acid was not converted to glutamic acid by liver extract. As the incubation time in Kumagai's study was too long (20 hours) and the crude enzyme solution was used, the authors repeated his experiment with partially purified enzyme.

* The compendium of this study were partly presented at the 20th Annual Meeting of Japanese Biochemists (1948) and the Medical Society of Osaka University (Feb. 1955).

* This study was supported by a grant for scientific research from the Ministry of Education. We should like to express our gratitude.

METERIALS AND MATHODS

Urocanic Acid—Obtained from histidine by deamination with histidine deaminase of the rabbit liver; m.p. 230–231°.

Enzyme Solution—L-Histidine monochlorhydrate was administered orally to the rabbit, weighing 2–3 kg, in a dosage of 0.5 g. per kg. of body weight. After 6 hours the animal was sacrificed by bleeding and the liver was ground with an equal amount of sea sand. The liver brei was extracted with 5 volumes of ice cold distilled water (pH 5.6) for one hour at 0–5° and centrifuged at 3000 r.p.m. for 15 minutes. The supernatant was adjusted to pH 5.2 with 10 per cent acetic acid and the precipitate was centrifuged at 4000 r.p.m. for 30 minutes. The supernatant possessed a high activity for decomposing urocanic acid but does not deaminate histidine. The crude enzyme solution was fractionated with $(NH_4)_2SO_4$ at pH 5.6 (optimal pH) and the fraction of 30–50 per cent (see Table II) was dissolved in minimal water (salted-out enzyme). The enzyme was adsorbed with 4 per cent volume of the solution of Ca_3-$(PO_4)_2$-gel at 0–5° for 30 minutes and centrifuged at 3000 r.p.m. for 10 minutes. The precipitate was washed until the wash water no longer gave a positive Nessler's and Pauly's reactions, and was then eluated with a volume of 1/15 *M* phosphate buffer (pH 8.4) equal to used for the adsorbtion and after 5–10 hours the solution was centrifuged at 3500 r.p.m. for 15 minutes. The supernatant was used as the purified enzyme (eluated enzyme).

Estimation of Urocanic Acid—Urocanic acid was estimated after Sera's method (*2*). The reacting solution was deproteinized with trichloroacetic acid and to 1–2 ml. of the filtrate was added 2 ml. of diazo reagent. After one minute, 3 ml. of 4 per cent NaOH was added and the total volume was made up to 8 ml. with water and mixed well. After keeping at 50° for 30 minutes, the yellow color was measured using Pulfrich's photometer with filter S_{47}.

Estimation of Oxygen-Uptake—This was carried out using Warburg's manometer as described later.

RESULTS

Activity of each Enzyme Fraction Fractionated with $(NH_4)_2SO_4$—The highest activity was indicated in the fraction of 30–50 per cent saturation (Table I).

Oxygen-Uptake in the Decomposition of Urocanic Acid—The experiments were carried out under the conditions shown in the Table II and oxygen-uptake was demonstrated in some cases, as shown in the Table III, but not in all cases.

After the content of the side chamber was poured into the main compartment, the reacting solution was incubated at 37° for 120 minutes.

As glutamic acid and formic acid indicated no O_2-uptake (Table

TABLE I

Enzyme Activity of Each Fraction Fractionated with $(NH_4)_2SO_4$

Fraction	Amount of decomposed urocanic acid (γ)	Rate of decomposition
%		%
0– 30	30	6
30– 50	500	100
50– 70	155	31
70–100	40	8

Conditions: 1.0 ml. of enzyme solution, 500 γ of the substrate (dissolved in 0.5 ml. of distilled water), 0.3 ml. of 1 *M* phosphate buffer (pH 6.2) and 1.2 ml. of distilled water were well mixed (pH 6.2) and incubated at 37° for 2 hours.

TABLE II

Composition in Each Vessel

Room	A	B	C	D	F
Main	0.3 ml. of 1 *M* phosphate buffer (pH 6.2)	"	"	"	"
	1.0 ml. of enzyme solution	"	"	"	"
	1.2 ml. of water	"	"	"	"
Side	3 μM Urocanic acid	3μM glutamic acid	3 μM sudium formate	Distilled water	Distilled water
Center	0.2ml. of 50% KOH	"	"	"	"

IV), the oxygen consumed in these experiments was used for the decomposition of urocanic acid.

Effect of Ethylendiamine Tetraacetate (EDTA)—As shown in Table IV, O_2-uptake was constantly observed with addition of EDTA and it was highest at $10^{-2}M$ of EDTA. As EDTA was more effective on the alkaline side than on the acid, all the following experiments were carried out at pH 8 and $10^{-2}M$ of EDTA.

Experiments with the Enzyme Solution Treated with Norit—Norit was added to the liver extract in concentration of 1/10 the total volume and the pH was adjusted to 4.9 with 10 per cent acetic acid and after the

TABLE III

Oxygen-Uptake in the Decomposition of Urocanic Acid

Substrate (Vessel) \ No. of cases	1	2	3	4	5	6
A. Urocanic acid (μM) was completely decomposed	μl. 35.2	μl. 35.6	μl. 30.9	μl. 37.9	μl. 32.9	μl. 27.2
Atom of oxygen taken up per mol. of substrate	1.05	1.06	0.92	1.13	0.98	0.81
B. L-Glutamic acid (3 μM)	0.00	0.00	0.00	0.00	0.00	0.00
C. Sodium formate (3 μM)	0.00	0.00	0.00	0.00	0.00	0.00
D. Enzyme alone	0.00	0.00	0.00	0.00	0.00	0.00

TABLE IV

Oxygen-Uptake of the Urocanic Acid Decomposition on Addition of EDTA

Concentration of EDTA	O_2-Uptake with each enzyme preparation					
	Salted-out		Dialyzed		Eluated	
	O_2	Oxygen atom per mol. urocanic acid	O_2	Oxygen atom per mol. urocanic acid	O_2	Oxygen atom per mol. urocanic acid
M	μl.	*atom*	μl.	*atom*	μl.	*atom*
0	0	0	0	0	0	0
10^{-4}	0	0	23.5	0.70	33.4	0.99
10^{-3}	4.6	0.14	33.9	1.02	42.5	1.26
10^{-2}	15.4	0.46	21.8	0.65	51.4	1.59

Urocanic acid: 3 μM, 37.5°, pH 8.0, reaction time 3 hours. Urocanic acid was completely decomposed.

solution was kept at 0–5° for 25 minutes, it was centrifuged at 10000 r.p.m. and 4° for 10 minutes. The supernatant was treated as described above. (Table V)

Experiment with the Rabbit Administered Aminopterine—Tabor *et al.* (*3*) isolated formamidinoglutaric acid from the urine of the folic acid deficient rat. The following data were obtained in the aminopterine

Table V

Urocanic Acid Decomposition by Oxygen-Uptake of the Enzyme Solution Treated with Norit, Isoelectric Precipitation and $(NH_4)_2SO_4$

pH	EDTA	O_2-Uptake							
		No. 1		No. 2		No. 3		No. 4	
		A	B	A	B	A	B	A	B
	M	*μl.*	*atom*	*μl.*	*atom*	*μl.*	*atom*	*μl.*	*atom*
6.1	0	37.6	1.12			29.8	0.89	33.5	1.00
	10^{-2}	37.2	1.11			29.9	0.89		
6.6	0	29.1	0.87						
7.1	0	18.7	0.56						
	10^{-2}	35.0	1.04						
7.6	0	25.3	0.75						
8.0	0	36.1	1.07	37.2	1.11	17.9	0.53	29.2	0.87
	10^{-2}	52.7	1.57	68.4	2.04	66.7	1.99	59.3	1.76

Note: A, O_2-uptake in; B, oxygen atom consumed per mole of urocanic acid. Urocanic acid was completely decomposed.

Condition: 3 μM of urocanic acid, reaction time 2–3 hours.

Table VI

Oxygen-Uptake of the Urocanic Acid Decomposition by the Enzyme Preparation Treated with Norit

Treatment of enzyme preparation	Addition of EDTA	O_2-Uptake	Oxygen atom consumed per mol. of urocanic acid
	M	*μl.*	*atom*
Supernatant of norit and isoelectric precipitation	0	0.0	0.00
	10^{-2}	35.2	1.05
Fraction of 30–50% of $(NH_4)_2SO_4$	0	37.2	1.11
	10^{-2}	68.4	2.04
Elution of Ca-phosphate-gel adsorption	0	19.1	0.57
	10^{-2}	34.3	1.02

Note: Urocanic acid was completely decomposed. Conditions were same as Table V.

administered rabbit. Rabbits weighing 1.8 kg. were given 0.5 mg. of aninopterine daily by intraperitoneal injection. After 40 days the animals was sacrificed and the liver was treated as described.

TABLE VII

Oxygen-Uptake of the Urocanic Acid Decomposition by the Liver Enzyme of Aminopterine Ijnected Rabbit

Enzyme preparation	Addition of EDTA	O_2-Uptake	Oxygen atom consumed per mol. of urocanic acid
	M	*μl.*	*atom*
Salted out	—	16.2	0.48
		37.5	1.12
Eluted	—	20.5	0.61
		39.0	1.16

Note: Urocanic acid was completely decomposed.
Condition: Urocanic acid 3 μM., final concentration of EDTA 2×10^{-3} *M*, temperature 37.5, time 2 hours.

DISCUSSION

Many reports have been published on the conversion of urocanic to glutamic acid and all suggest the hydrolytical pathway of degradation. As histidine is deaminated to urocanic acid without O_2-uptake (*1*), no oxygen is consumed in the entire route from histidine to glutamic acid.

It was proven in this laboratory about 10 years ago (*1*), that oxygen was consumed in the degradation of urocanic acid by the liver extract. However, the O_2-uptake was not constant; sometimes one atom of oxygen, sometimes much less and sometimes no oxygen was shown to be consumed. We have been frequently criticized by other investigetors, that in the decomposition of urocanic acid, oxygen was never consumed.

EDTA, Norit or aminopterine were used in the experiments as described above and better results than in the earlier studies were obtained. Much better results were obtained using Pseudomonas aeruginosa, as will be shown in following papers.

SUMMARY

1. Urocanic acid is decomposed by enzyme, prepared from the rabbit liver, under O_2-uptake. This fact became clear by the addition of EDTA to the enzyme solution.

2. When the liver extract was treated with Norit, the partially purified enzyme solution decomposed urocanic acid oxidatively without EDTA.

3. The liver extract of rabbit, administered 0.5 mg. aminopterine daily by injection for 40 days, decomposed urocanic acid oxidatively without EDTA.

REFERENCES

(*1*) Kumagai, N., The 20th Annual Meeting of the Society of Japanese Biochemists (1948)
(*2*) Sera, Y., *J. Osaka Med. Soc.*, **38**, 1021 (1939)
(*3*) Tabor, H., Silverman, M., Mehler, A. H., Daft, F. S., and Bauer, H., *J. Am. Chem. Soc.*, **75**, 755 (1953)

The Journal of Biochemistry, Vol. 43, No. 5, 1956

STUDIES ON THE OXIDATIVE DECOMPOSITION OF UROCANIC ACID BY PSEUDOMONAS AERUGINOSA. II*

By SHOZO OTA, TSUTOMU WACHI, MAKIO UCHIDA, YUKIYA SAKAMOTO and KATASHI ICHIHARA

(*From the Biochemical Department, Medical School, Osaka University, Osaka*)

(Received for publication, April 28, 1956)

In the previous paper (*1*) it was confirmed that urocanic acid was decomposed by the liver enzyme of rabbit when EDTA was added to the reacting solution with the consumption of 1–2 atoms of oxygen per molecule of the substrate. The following experiments were carried out with *Pseudomonas aeruginosa*.

MATERIALS AND METHODS

Enzyme preparation of Pseudomonas aeruginosa—The strain was cultured in neutral bouillon, to which L-histidine chlorhydrate was added in a concentration of 4 per cent, under shaking at 37° for 18 hours. The cells were collected by ultracentrifuge (Sharpless) and washed with 0.9 per cent saline solution until the wash solution no longer gave a positive diazo reaction. The cellbrei was suspended in minimal distilled water and ten times the volume of ice cold acetone was added and the solution filtered through a Buchner's funnel. The cell mass was dried in vacuum at low temperature. The cell powder was ground with two times the weight of quartz sand and extracted with *M*/15 phosphate buffer (25 ml. to 1.0 g. cell) at 0–5° for one hour and centrifuged at 4000 r.p.m. for 30 minutes. The supernatant was treated in the same manner as the procedure for enzyme preparation, described in the previous papers: isoelectric precipitation, salting out at 30–50 per cent saturation of $(NH_4)_2SO_4$, adsorption with $Ca_3(PO_4)_2$-gel and elution. The eluate was dialyzed against distilled water through collodion membrane at 0–5° for 18 hours.

Determinations of Histidine, Urocanic Acid and Glutamic Acid—These were determined according to Sera's (*2*), Sera's (*3*) and Robert's (*4*) methods, respectively.

---

* The compendium of this study were partly announced at the Symposium of Enzyme Chemistry (1951) and the Medical Society of Osaka University (Feb. 1955).

* This study was supported by a grant for scientific research from the Ministry of Education. We should like to express our gratitude.

Measurement of O_2-Uptake—O_2 was measured using Warburg's manometer.

Qualitative Analysis of Metals—The metals present in trace were proven after Kikkawa and Ogita's method (*5*) by means of paperchromatography after ashing the sample.

Contents of the Warburg's Vessel—Unless otherwise specified, each chamber contained the following:

Main compartment: 0.3 ml. of 1 *M* phosphate buffer (pH 8.0), 1.0 ml. of enzyme solution and addenda. The total volume was made up to 3.0 ml. by addition of distilled water.

side chamber: Urocanic acid (3–6 μM) was dissolved in 0.5 ml. of distilled water.

Second side chamber: 0.1 ml. of 25 per cent H_2SO_4. This was used only for the measurement of CO_2.

Center well: 0.1 ml. of 50 per cent KOH.

The reaction was carried out at pH 8.0, 37.5° for 2–3 hours.

RESULTS

Experiments with Acetone-Dried Cells—As shown in Table I, the fresh acetone-dried cell decomposed urocanic acid without O_2-uptake or with minimal uptake but when the cell powder was kept in the refrigerator for about two weeks, it decomposed urocanic acid under uptake of about one atom of oxygen. By the addition of EDTA, the O_2-uptake increased to two atom per molecule of urocanic acid.

TABLE I

Oxygen-Uptake for the Decomposition of Urocanic Acid (Cell Powder)

Acetone-dried cell	EDTA	O_2-Uptake	Urocanic acid decomposed	Oxygen atom consumed per mole urocanic acid
Fresh	—	0.0 *μl.*	100 *%*	0.00 *atom*
Stored	—	21.1	96	0.65
	—	27.6	100	0.82
	+	64.9	95	2.00

Fresh designates cells taken soon after drying with acetone, stored designates cells, kept in the refrigerator for two weeks. 20 mg. of cell was suspended in 1.0 ml. of *M*/15 EDTA was added in final conct. of 10^{-3} *M*.

When the O_2-uptake came to a standstill, glutamic acid was no longer formed (Table II).

Experiments with Cell Extract—The most effective final concentration

TABLE II

Relationship between Oxygen-Uptake and Glutamic Acid Formation by the Stored Cell Powder

After O_2-Uptake was still	O_2-Uptake	Urocanic acid decomposed	Oxygen atom consumed per mole urocanic acid	Glutamic acid formed
	μl.	*%*	*atom*	*M*
Soon	27.6	100	0.82	0.27
1 hour	35.3	100	1.02	0.33
2 „	28.6	100	0.85	0.26

As glutamic acid and formic acid did not show O_2-uptake with the acetone-dried powder, the oxygen consumed must have been used for the decomposition of urocanic acid. The slight formation of glutamic acid suggested that urocanicase was not completely lost in this preparation.

of EDTA for the O_2-uptake and urocanic acid decomposition was 10^{-3} *M*. (Table III)

TABLE III

Optimal Concentration of EDTA (Extract)

Final concentration of EDTA	O_2-Uptake	Urocanic acid decomposed	Oxygen atom used per mole of uroc. acid
M	*μl.*	*%*	*atom*
None	37.0	100	0.6
2×10^{-3}	130.4	100	2.0
10^{-3}	134.1	100	2.0
10^{-4}	101.7	100	1.5
10^{-5}	64.7	100	1.0
10^{-6}	52.8	100	0.8

As shown in Table IV, O_2-uptake still continued even after urocanic acid was decomposed 100 per cent and after 2–3 hours the maximum was reached. (Table II). This fact is very interesting, because it means that the nonoxidative decomposition of urocanic acid precedes the oxidative or the oxidation takes place by step.

As in the case of the acetone-dried cell, the extract prepared from fresh dried-cell decomposed urocanic acid under much less oxygen uptake than that prepared from stored cell. By the addition of EDTA, however, both extracts decomposed urocanic acid with consumption of about two

TABLE IV

Relationship between the Oxygen-Uptake and the Decomposition of Urocanic Acid. (Extract)

Reacting time	O_2-Uptake	Decomposition of urocanic acid	Oxygen atom used per mole urocanic acid
mm.	*μl.*	*%*	*atom*
20	21.0	78	0.8
40	44.2	100	1.32
60	52.1	100	1.56
90	64.3	100	1.94

EDTA: 10^{-3} *M.*

atom of oxygen per molecule of the substrate. On the contrary, the formation of glutamic acid was much greater without addition of EDTA, especially in the case of the fresh cells (Table V).

TABLE V

Oxygen-Uptake and Glutamic Acid Formation by the Extracts Prepared from Fresh Dried and Stored Cells

Extract from	EDTA	O_2-Uptake	Decomposition of glutamic acid	Oxygen atom used per mole of urocanic acid	Glutamic acid formed
		μl.	*%*	*atom*	*M*
Fresh	−	9	100	0.3	1.0
Dried cell	+	59.3	100	1.8	0.2
Stored	−	34.3	100	1.0	0.4
cell	+	67.1	100	2.0	0.2

Of the metal ions tested, only Cu ion inhibited the effect of EDTA (Table VI).

Experiments with Salted-out Enzyme Solution—As shown in Table VII, glutamic acid was not formed by the salted-out enzyme solution, either with or without the addition of EDTA. Among the compounds tested, oxine, $\alpha\alpha'$-dipyridyl and EDTA increased the O_2-uptake and Ag^+ and Hg^{++} inhibited the decomposition of urocanic acid completely.

Experiments with the Eluted and Dialyzed Enzyme Solutions—While O_2-uptake was increased to about two atoms by the addition of EDTA with the salted-out enzyme solution, it was maintained at about one

Table VI

Inhibition of Metal Ions

EDTA	Metal ion added	O_2-Uptake	Decomposition of urocanic acid	Oxygen atom used per mole of urocanic acid
		μl.	*%*	*atom*
None	None	40.6	96	0.6
	None	121.4	96	1.8
	Co	79.8	94	1.2
	Mn	139.2	88	2.1
Added in 10^{-3} *M*	Mg	130.7	98	1.9
	Cd	104.6	98	1.6
	Zn	126.5	98	1.9
	Fe	112.0	96	1.7
	Cu	46.2	74	0.7
	Ca	102.0	93	1.5

6 μM of urocanic acid; 2×10^{-3} *M* of metal ion in finalconc.; Reaction time 150 minutes.

Table VII

Influence of Various Compounds on the Decomposition of Urocanic Acid Oxygen-Uptake by the Salted-out Enzyme Preparation

Addition	O_2-Uptake	Decomposion of uroc. acid	Oxygen atom consumed per mole uroc. acid	Glutamic acid formed
	μl.	*%*	*atom*	*mole*
None	26.2	100	0.8	0
EDTA	59.8	95	1.9	0
Oxine	33.6	71	1.4	
α,α′-Dipyridyl	67.2	97	2.1	
NaN_3	34.3	98	1.0	
$Na_2S_2O_3$	12.8	98	0.4	
CH_2JCOOH	33.6	99	1.0	
NaF	26.2	99	0.8	
Ag_2SO_4	0.0	0	0.0	
$HgCl_2$	0.0	0	0.0	
Formate	21.2	100	0.6	
Semicarbazide	16.6	100	0.5	
Malonic acid	22.2	100	0.7	
Arsenic acid	22.7	100	0.7	
Fumaric acid	21.0	100	0.6	
Benzoic acid	23.1	100	0.7	
NH_4Cl	23.5	100	0.7	
Hydroxylamine	0.0	54	0.0	
NaCN	33.8	100	1.0	
Sulfamine	28.1	98	0.9	
PCMB	33.7	98	1.0	

3 μM of urocanic acid; 10^{-3} *M* of each addition in final cocn.; Reaction time 90 minutes; PCMB *p*-Chloromercuric benzoate.

atom even by the addition of EDTA with the eluted and dialyzed enzyme solutions (Table VIII).

When cell extract was newly added to the reacting solution, after the O_2-uptake by the dialyzed enzyme had come to a standstill, one more atom of oxygen was found to be consumed either with or without the addition of EDTA (Table IX). Glutamic acid was not formed in this case.

TABLE VIII

Oxygen-Uptake by the Purified Enzyme

Enzyme preparation	EDTA	O_2-Uptake	Decomposition of uroc. acid	Oxygen atom consumed per mole uroc. acid
		μl.	*%*	*atom*
Eluted	−	46.0	100	1.37
	+	23.4	100	0.69
Dialyzed	−	32.1	100	0.96
	+	28.8	100	0.86

TABLE IX

O_2-Uptake by the Alternative Addition of the Dialyzed and Extracted Enzyme Preparation

Enzyme solution	O_2-Uptake by the dialyzed enzyme	O_2-Uptake by the newly added extract	Decomposition of uroc. acid	Oxygen atom consumed per mole uroc. acid.	Glutamic acid formed
	μl.	*μl.*	*%*	*atom*	*mole*
Dialyzed	36.4		80	1.37	0
Dialyzed +extract	40.0	12.2	83	1.88	0
Dialyzed+extract+EDTA	35.6	24.3	88	2.00	0

Urocanic acid: 3 μM. Reaction time: 200 min.

DISCUSSION

It became clear from experiments with *Pseudomanas aeruginosa*, that the discrepancies in the results, which had perplexed us up to now, was a result of the disregard of the freshness of the enzyme preparation. When the acetone-dried cell was used soon after preparation of the

cell powder, O_2-uptake was not shown, but after the powder was kept in the refrigerator for two weeks, about one atom of oxygen was consumed per molecule of urocanic acid (Table I). The same results were also found in the case of the cell extracts which were obtained from the fresh and store powders (Table V). Without EDTA, one atom of oxygen was consumed and with addition, O_2-uptake was increased to two atoms per molecule of urocanic acid.

Marked formation of glutamic acid was noted only in the cases of low O_2-uptake (Table V) and after the oxygen consumption came to a standstill, the glutamic acid was no longer formed (Table II). On the contrary, even in with 100 per cent disappearance of urocanic acid, O_2-uptake gradually increased with time and approximated two atoms per molecule of the substrate (Table IV). Glutamic acid was formed by the cell powder and the cell extract but not by the salted-out (Table VII), the eluted or the dialyzed enzyme preparation (Table VIII). When cell extract was newly added, after the dialyzed enzyme had acted on the substrate sufficiently, further O_2-uptake was shown and irrespective of the presence of EDTA, almost two atoms of oxygen were consumed per molecule of urocanic acid. It is needless to state that glutamic acid oxidase, formic acid oxidase and catalase were shown to be absent in our enzyme preparation.

The facts suggest that: the oxidative pathway of urocanic acid is different from the pathway to glutamic acid and the enzyme for the latter can be removed by salting-out with $(NH_4)_2SO_4$, or more completely by treatment with Ca-phosphate gel. One of the two atoms of oxygen consumed for the decomposition of urocanic acid is affected by EDTA etc. (Table VII) and this metal catcher sensitive part can be removed by dialysation of the eluted enzyme solution.

Recently, succinic monoureide and succinic acid were isolated as the oxidative decomposition product of urocanic acid in the precence of EDTA to the extract of *Pseudomonas aeruginosa* (*6*).

SUMMARY

1. When the acetone-dried powder of *Pseudomonas aeruginosa* was kept for a long time in the refrigerator, the pathway from urocanic acid to glutamic acid was lost and the oxidative pathway appeared. In the latter, one atom of oxygen was consumed and by the addition of EDTA one further atom was taken up.

2. Enzyme for the oxidative decomposition of urocanic acid was

partially purified from *Pseudomonas*. The purified enzyme did not form glutamic acid but decomposed urocanic acid under uptake of one atom of oxygen either with or without addition of EDTA. This indicated, that the substance sensitive to EDTA, was removed by the procedure of enzyme preparation.

3. Oxine and α,α'-dipyridyl were proven to be as effective as EDTA but Ag^{+} and Hg^{++} inhibited the decomposition of urocanic acid completely. Hydroxylamine was an exception and 54 per cent of the urocanic acid was decomposed by it without O_2-uptake.

4. Among the metal ions, only Cu^{++} inhibited the effect of EDTA.

REFERENCES

(*1*) Ichihara K., Itagaki S., Suzuki Y., Uchida M., and Sakamoto Y., *J. Bichem.*, **43**, 603 (1956)
(*2*) Sera, Y., *J. Osaka Med. Soc.*, **38**, 1029 (1939)
(*3*) Sera, Y., *J. Osaka Med. Soc.*, **38**, 1021 (1939)
(*4*) Ayengar, P., Roberts, E., and Ramasarma, G. B., *J. Biol. Chem.*, **193**, 781 (1951)
(*5*) Kikkawa, H., and Ogita Z., *Kagaku*, **10**, 77 (1955)
(*6*) Ichihara, K., Sakamoto, Y., Satani, H., Okada, N., Kakiuchi, S., Koizumi, T., and Ota, S., *J. Biochem.*, in press.

The Journal of Biochemistry, Vol. 43, No. 5, 1956

7-HYDROXYINDOLE DERIVATIVES AND THE ACID DIAZO-REACTION

By KATASHI ICHIHARA and YUKIYA SAKAMOTO

(*From the Biochemical Department, Medical School Osaka University, Osaka*)

(Received for publication, April 28, 1956)

Three types of diazo-reactions were applied in our biochemical studies; namely Pauly's, Ehrlich's and Sera's diazo-reaction. The Pauly's diazoreaction, in which the solution was coloured red by Na_2CO_3, was used to detect phenol and imidazol derivatives. In Ehrlich's diazo-reaction the foam of the solution became red by addition of NH_4OH and the so-called urochromogen was proved. Recently Makino (*1, 2*) reported, that the Ehrlich's diazo-positive substances in the urine of tuberculous patients were 3-hydroxykynurenine and xanthurenic acid. In the Sera's diazo-reaction (*3*) 4 per cent NaOH was used for alkalization and the solution was coloured yellow. Sera devised a method of colorimetric estimation of urocanic acid, based on this reaction. Furthermore he observed, that in the degradation of urocanic acid by the liver extract, a substance was formed, which gave a red colour in his diazo-reaction. In spite of his long effort, this substance has not yet been isolated, though 4-oxoimidazole compound was assumed by Japanese authors, who studied the degradation of urocanic acid. Hydantoinpropionic acid did not give this reaction.

About 26 years ago Ichihara and Yoshida (*4*) administered scatolylhydantoin (tryptophanehydantoin) orally to the rabbit and found that the urine gave a red diazo-reaction without addition of alkali. As sulfanilic acid is dissolved in 3.3 per cent of HCl, the authors wish to designate this type of diazo-reaction as the acid diazo-reaction. As the acid diazo-reaction was given only by 7-hydroxyindole derivatives, as shown in the following table, the substance in the urine of the scatolylhydantoin administered rabbit must be a 7-hydroxytryptophan derivative. The biological oxidation at the 5-position of tryptophan derivatives was confirmed by the bufotoxius and serotonine and the oxidation at the 7-position was demonstrated in this experiment. The chemical oxidation of tryptophan at the 5- and 7-positions has been already shown by Dalgliesh (*5*), using EDTA, Fe^{++} and vitamin C. The solution thus

obtained was also found to give the acid diazo-reaction.

MATERIALS AND RESULTS

	Acid diazo-reaction	Ehrlich's aldehyde reaction	$FeCl_3$
5-Hydroxyindole ethylamine (serotonine)	−	+	reddish brown
5-Hydroxytryptophane	−	+	brown
8-Hydroxyquinoline (oxine)	−	−	green
4,8-Dihydroxyquinoline-α-carbonic acid (xanthurenic acid)	−	−	blue
7-Hydroxyindole*	+	+	reddish violet
7-Hydroxyindole acetic acid	+	+	green
Chromatographic spot of the urine of rabbit, administered scatolyhydantoin	+	+	green
Solution of tryptophan oxidized by Dalgliesh's method	+	/	/

* 7-Hydroxyindole gave a red colour with HNO_2, but this reaction differered from the acid diazo-reaction. The acid diazo-reaction was also positive, when diazobenzolonium sulfonic acid, made free from HNO_2, was used.

3-Hydroxyanthranilic acid, 3-hydroxykynurenine, kynurenic acid, pyrrole-α-hydroxytryptophan were negative. The urine of rabbit, administrated pyrrole-α-methylscatolyl hydantoin, did not give this reaction. The extract of the bufo skin gave positive spot.

Paperchromatography—The urine of the rabbit, administered 0.5 gm. of scatolylhydantoin, was extracted 5 times with equal volumes of ethylacetate and the ethylacetate was evaporated off on the water bath to give a syrup. The syrup was extracted with minimal amount of absolute alcohol and the alcohol extract was used for the paperchromatography. The developing solution was a mixture of *n*-butanol, acetic acid and distilled water (4 : 1 : 5). A spot was revealed at Rf 0.59 by spraying the diazo-reagent, Ehrlich's aldehyde reagent and $FeCl_3$. This results indicate that the benzene ring of the indole nucleus was oxidized and the pyrrole moiety remained intact.

7-Hydroxyindole, 5-hydroxytryptophan and 7-hydroxyindole acetic acid were

supplied by Dr. O. Hayaishi, Dr. Y. Takagi and Prof. T. Sakan respectively. We should like to express our hearty thanks.

REFERENCES

(*1*) Makino, K., Satoh, K., Fujiki, T., and Kawaguchi, K., Nature, **170**, 977 (1952)
(*2*) Makino, K., and Takahashi, H., *J. Biochem.*, **42**, 1, (1955)
(*3*) Sera, Y., Nakamuro, K., Tsuda, I., and Sonoda, K., *Med. J. Osaka Univ.*, **38**, 1017 (1938)
(*4*) Ichihara, K., and Yoshida, Y., *Med. J. Osaka Univ.*, **30**, 2413 (1930)
(*5*) Dalgliesh, C. E., *Arch. Biochem. Biophys.*, **58**, 214 (1955)

The Journal of Biochemistry, Vol. 43, No. 5, 1956

SOME PROPERTIES OF A BACTERIAL GLUCOKINASE*

By RYO SATO, SHIGEKI TAKEMORI** and MITUO EBATA

(From the Department of Chemistry, Faculty of Science, Kanazawa University, Kanazawa)

(Received for publication, May 7, 1956)

Metabolic attack in most organisms on hexoses appears to be commenced by phosphokinase reactions in which the terminal phosphate radical of adenosine triphosphate (ATP) is transferred to the free sugar molecules. Crystalline yeast hexokinase, one of the best characterized enzymes of this class, catalyses the phosphorylation of glucose, fructose and mannose (*2*, *3*) as well as of D-glucosamine (*4*) to form the corresponding 6-phosphates. A hexokinase closely resembling the yeast enzyme has also been reported to occur in brain (*5*). In the case of muscle and liver, on the other hand, it has been shown that glucose and fructose are phosphorylated by different enzymes which could be obtained separately in different protein fractions (*6*). The specificity of the glucokinase has not yet been examined and it has not been difinitely shown that the product is glucose-6-phosphate. The fructokinase of liver has been partially purified (*7*, *8*) and the product shown to be fructose-1-phosphate (*6*, *9*).

Studies on the corresponding systems in bacteria have also produced evidence that different kinases are responsible for the phosphorylation of glucose and fructose. Klein and Doudoroff (*10*) have described a glucokinase of *Pseudomonas putrefaciens* which is inactive to fructose. Cardini (*11*) separated glucokinase of *Escherichia coli* from fructokinase, but found it still active to mannose. Glucokinase of *Staphylococcus aureus*, on the other hand, had no activity towards either fructose or mannose (*11*). In all these studies on bacterial enzymes, however, only a little effort has been made to characterize their properties in detail.

The purpose of this paper is to describe some fundamental properties of a specific glucokinase which has been found in a *Bacillus* species

* This paper was read at the 9th Annual Meeting of the Chemical Society of Japan, Kyoto, April 2, 1956 (*1*).

** Present address: Department of Biology, Faculty of Science, Osaka University.

designated as strain W-2. This organism is the same with what we employed in a study (*12*, *13*) on an adaptive enzyme capable of phosphorylating sedoheptulose.

EXPERIMENTAL

Materials and Methods

Unless otherwise stated, strain W-2 was grown at 35° for 20–22 hours on an agar medium containing 0.1 per cent glucose. The composition of the medium was as follows: peptone, 10 g.; meat extract, 10 g.; NaCl, 3 g.; glucose (autoclaved separately), 1 g.; agar, 25 g.; distilled water, 1,000 ml.; pH adjusted to 7.0–7.2. Cell-free extracts containing glucokinase were prepared and partially purified from the cells by exactly the same method as employed in the extraction of the sedoheptulose kinase from the heptose-adapted cells of the same organism (*13*). The enzyme preparation thus obtained usually contained 0.3–0.6 mg. of Kjeldahl nitrogen per ml.

The glucokinase activity was followed by determining the decrease in acid-labile phosphate (hydrolysable in 7 minutes in 1 *N* HCl at 100°) as described for the sedoheptulose enzyme (*13*). Control experiments without sugars were also run at the same time. In experiments in which the washed cells were used the disappearance of free glucose was followed as a measure of glucokinase activity; an 1 ml.-portion of the deproteinized (trichloracetic acid) reaction mixture was treated with 0.2 ml. of 25 per cent barium acetate solution, neutralized to pH 8.0 with KOH, and diluted to 10 ml. with 95 per cent ethanol. The precipitate of barium salts were then removed by centrifugation, and the glucose content of the supernatant was estimated colorimetrically using dinitrosalicylic acid reagent (*14*).

Sugars other than sedoheptulose used in the present study were all commercial products of high purity which were further purified by recrystallization. Their purities were checked by paper chromatography using several solvent systems and detecting reagents. The preparation of sedoheptulose was the same with that described previously (*13*). Sodium salt of ATP having a purity of 89 per cent was used.

RESULTS

Effect of Glucose Concentrations in Media on Glucokinase Production—In order to search for an optimal condition for the production of glucokinase, strain W-2 was grown on agar media prepared as described under "Materials and Methods" except that glucose concentrations were varied from nil to 0.6 per cent. After cultivation for 20 hours, the cells were harvested, washed twice with 0.8 per cent NaCl solution and once with distilled water, and finally suspended in distilled water. The activity of each batch of the washed cells to utilize glucose was then examined by determining the disappearance of the free sugar. The re-

sults which are illustrated in Fig. 1 indicated that those cells which were derived from cultures grown on 0.1 per cent glucose had a maximal activity. Above this concentration glucose exhibited an unfavorable effect on the enzyme production. In contrast to sedoheptulose-metabolizing system of the same bacterium (*12*), the production of glucokinase was appreciably high even if no glucose was added to the medium. This enzyme could, therefore, be classified into the so-called " constitutive " enzymes.

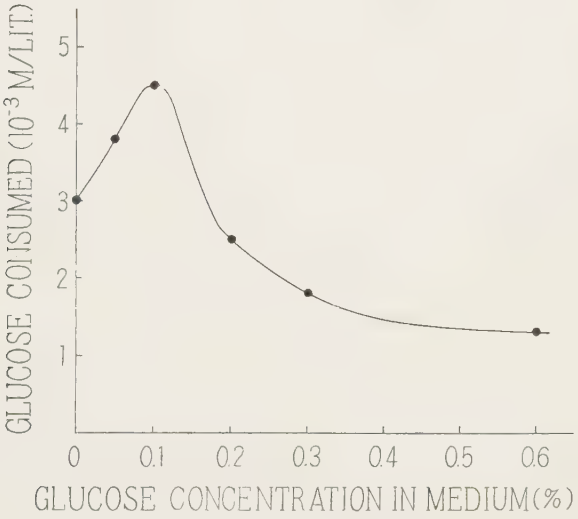


FIG. 1. Effect of glucose concentrations in media on glucokinase production.

Cells grown on media containing glucose at concentrations indicated were collected separately, washed, and suspended in distilled water. Each reaction mixture contained: 1 ml. of washed cell suspension (dry weight, 20–30 mg.), 1 ml. of 0.1 *M* glucose, 1 ml. of 0.01 *M* $MgCl_2$, and 1 ml. of 0.1 *M* phosphate buffer (pH 7.0). The mixtures were shaken in cotton-plugged bottle at 35° for 90 minutes. The results were expressed as amounts of glucose consumed by 5 mg. (dry weight) of cells.

Glucokinase Activity of Cell-free Preparation—Cell-free preparations obtained from the cells grown on 0.1 per cent glucose were then examined for glucokinase activity. When such a preparation was in-

cubated with glucose and ATP in the presence of Mg^{++} a remarkable decrease in acid-labile phosphate was observed (Fig. 2), a fact clearly demonstrating the phosphorylation of the sugar. Little reaction, however, took place in the absence of glucose. The possibility that the decrease in acid-labile phosphate in the complete system was due to the more hydrolysis of ATP was, therefore, excluded. Neither ATPase nor apyrase seems to be actively functioning in the system under the conditions employed.

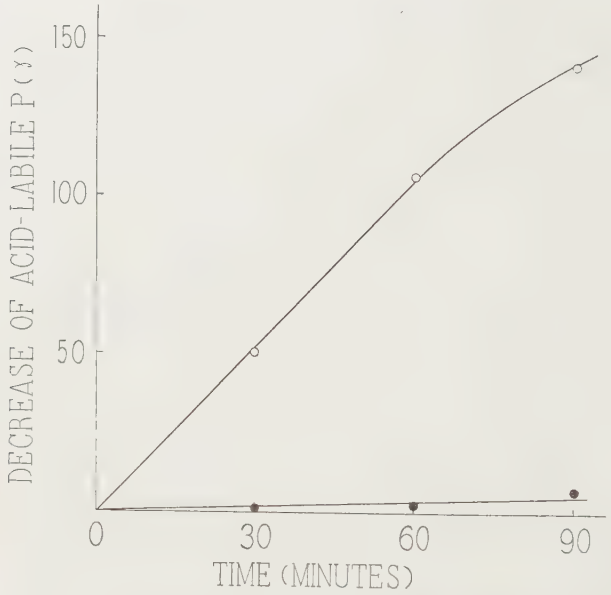


FIG. 2. Glucokinase activity of cell-free preparation.

Complete system (○) contained 2×10^{-3} *M* glucose, 2×10^{-3} *M* ATP, 1×10^{-2} *M* $MgCl_2$, 2×10^{-2} *M* citrate buffer of pH 6.9, and enzyme preparation containing 0.6 mg. N in a final volume of 5 ml. The composition of the control system (●) was the same except that glucose was omitted. Reaction was carried out in a open test tube. Temperature was 35°.

Effect of pH—The effect of pH on glucokinase activity was studied using citrate (pH 6–7.3) and veronal (pH 7.4–9.5) buffers. As is shown in Fig. 3, the activity was observed in a rather wide range of pH values.

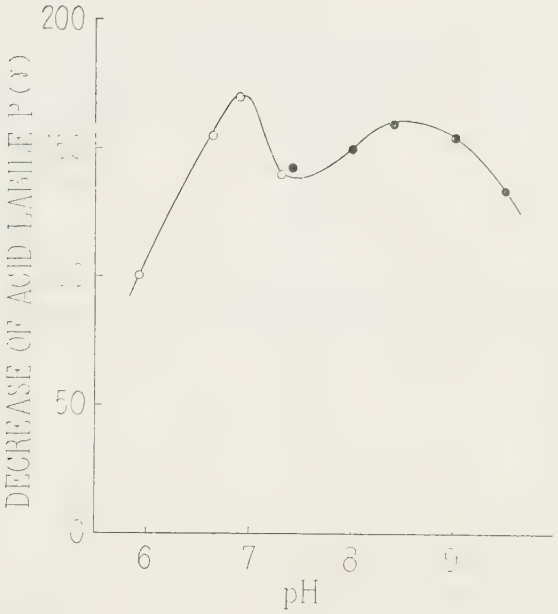


FIG. 3. Effect of pH.

Experimental conditions were the same as in Fig. 2, except that veronal (●) or citrate (○) buffer of the indicated pH values was used at a final concentration of 2×10^{-2} *M*. Reaction period, 90 minutes. Enzyme concentration, 0.5 mg. N per tube.

A striking feature of the pH-curve was, however, the fact that it showed two maxima at about pH 6.9 and 8.4 ; the latter maximum being broader and somewhat lower.

Metal Requirements—The enzyme activity has absolute requirements for metal cofactors. Table I shows that no activity was detected in the preparation without added metal ions. Among the metals tested, Mg^{++} and Mn^{++} were effective activators as in the case of most other phosphokinases. As will be seen from Fig. 4, Mg^{++} and Mn^{++} exhibited maximal activations at concentrations of 1×10^{-2} *M* and 8.3×10^{-3} *M*, respectively, when 2×10^{-3} *M* of ATP was used. The ratio of the optimal concentration of metal to ATP concentration used was 5 for Mg^{++} and 4.2 for Mn^{++}. Mg^{++} was about two times more effective as activator than Mn^{++} when compared at their respective optimal concentrations (Table I).

TABLE I

Efficiencies of Metals as Glucokinase Activators

Metal	Concentration (M)	Glucokinase activity (Relative value)
		%
Mg^{++}	1.0×10^{-2}	100
Mn^{++}	8.3×10^{-3}	54
Nil	—	0

Figures were taken from Fig. 4. The experiment without added metal ion was carried out under the same conditions except that metal ion was withdrawn from reaction mixture.

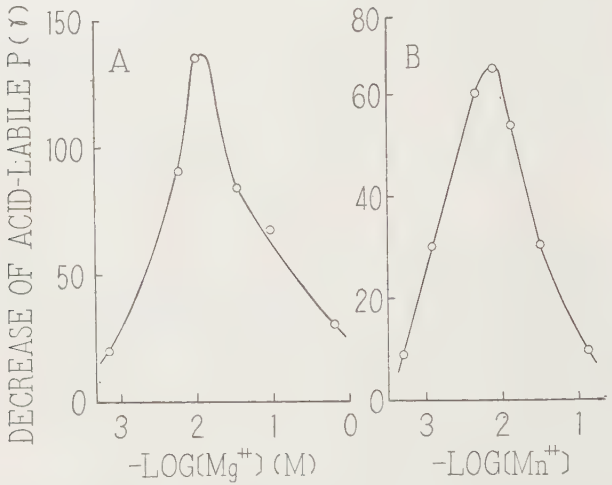


FIG. 4. Effects of Mg^{++} (A) and Mn^{++} (B) concentrations.

Experimental conditions were the same as in Fig. 2, except that Mg^{++} or Mn^{++} concentration was varied as indicated. Reaction period, 90 minutes. Enzyme concentration, 0.6 mg. N per tube.

Inhibition—In Table II are summarized the effects of several inhibitors. The inhibitions observed in this system are of almost the same magnitude with those found with sedoheptulose phosphokinase of the same organism (*13*) except that azide causes considerably less effect on the glucose enzyme. The inhibitions caused by $CuSO_4$, $HgCl_2$, iodoacetate, and particularly by *p*-chloromercuribenzoate suggested that

TABLE II

Inhibitions of Glucokinase

Inhibitor	Concentration (M)	Per cent inhibition
$CuSO_4$	1×10^{-2}	90
"	1×10^{-3}	62
$HgCl_2$	1×10^{-2}	100
"	1×10^{-3}	95
Iodoacetate	1×10^{-2}	94
"	2×10^{-3}	71
p-Chloromercuribenzoate	1×10^{-4}	97
"	1×10^{-5}	95
NaN_3	1×10^{-2}	25
"	1×10^{-3}	19
NaF	2×10^{-3}	14
"	1×10^{-3}	0

Experimental conditions were the same as in Fig. 2, except that inhibitors were added to reaction mixtures at concentrations indicated. Reaction period, 90 minutes. Enzyme concentration, 0.3 to 0.6 mg. N per tube.

the enzyme is a thiol enzyme. This is also in accord with the view that the dependence for activity on thiol groups is a general property of all phosphokinases (*15*). In common with a number of phosphokinases (*13, 16*), this enzyme was also found to be inhibited by alloxan as is shown in Fig. 5. This inhibition could be reversed by cysteine in a concentration equal to that of alloxan.

Specificity—Table III is a summary of the data obtained on the specificity of the enzyme preparation. While glucose was rapidly phosphorylated in this system, fructose was completely inactive. It was for this reason that we preferred to designate the enzyme as glucokinase rather than to call it hexokinase. Among the other sugars examined, mannose, galactose and L-arabinose also responded to a small but definite extent. The rate of mannose phosphorylation was one tenth of that of glucose phosphorylation. Galactose and L-arabinose reacted at the rates of 8 and 15 per cent, respectively, of that for glucose. In addition to fructose, D-glucosamine, D-ribose, D-xylose and sedoheptulose were completely inactive.

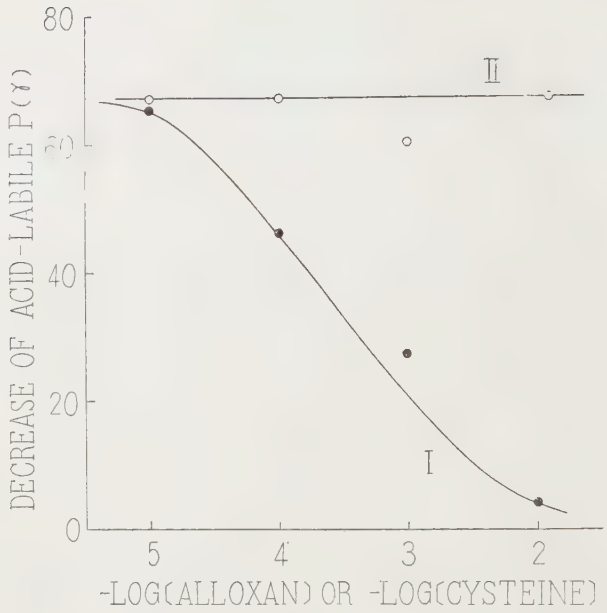


FIG. 5. Inhibition by alloxan and its reversal by cysteine.

In the inhibition experiments (●) alloxan was added at concentrations indicated. The reversal of inhibitions (○) was carried out in the presence of both alloxan and cysteine at equimolar concentrations indicated. The other conditions were the same as in Fig. 2. Reaction period, 90 minutes. Enzyme concentration, 0.4 mg. N per tube.

DISCUSSION

The results described in this paper indicate that the cell-free preparations obtained from strain W-2 contain a glucokinase but are devoid of fructokinase activity. This provides additional evidence for the general tendency that different enzymes are concerned in bacterial phosphorylation of glucose and fructose.

Among glucokinases of bacterial origin hitherto reported, *E. coli* enzyme (*11*) seems to be similar to the present one in that the both systems phosphorylate mannose as well as glucose. The affinity of the present enzyme towards mannose is, however, considerably low. This enzyme differs from glucokinase of *S. aureus* (*11*) which has no activity

TABLE III

Substrate Specificity of Glucokinase

Substrate	Relative activity
Glucose	100
Fructose	0
Mannose	10
D-Glucosamine	0
Galactose	8
L-Arabinose	15
D-Ribose	0
D-Xylose	0
Sedoheptulose	0

Experimental conditions were the same as in Fig. 2, except that various substrates indicated were added in place of glucose at final concentrations of 2×10^{-3} *M*. Reaction period, 90 minutes. Enzyme concentration, 0.4 mg. N per tube.

towards mannose. It is also different from *P. putrefaciens* enzyme (*10*, *17*), since the latter has a marked affinity towards D-glucosamine which is inert in this system. It is, however, not easy to establish the identity of these enzymes at the present stage of knowledge, since none of them has been sufficiently purified to permit such discussions. It is probable that the activity of these systems towards different sugars may be accounted for by the presence of more than two different enzymes.

The two peaks observed in the pH-activity curve (Fig. 3) may require some comments. It is, of course, possible that such a pH-curve is caused by the presence of two different glucokinases of different pH-sensitivity. Since our preparation is still rather crude, we have at present no arguments against this possiblity. It is, however, interesting to note that such double-peaked pH-curves have also been obtained with other phosphokinase systems such as sedoheptulose kinase of the same organism (*13*), sedoheptulose-utilizing system of spinach leaves (*18*), phosphohexokinase from peas (*19*) and hexokinase from wheat germs (*20*). The pH-curves of these enzymes, except for that of wheat germ hexokinase, are apparently similar to each other in that they possess, in common, higher and sharper maxima at their acid side. Accordingly, it may also be a possiblity that the existence of two pH optima is

a general property of a certain class of phosphokinases. The mechanism underlying this unusual phenomenon is, however, to be clarified.

No attempts have yet been made to isolate and identify the product of glucose phosphorylation. It does not appear likely, however, that the product is glucose-1-phosphate, since the phosphate radical of this ester is hydrolysable in 7 minutes in 1 *N* HCl at 100° (*21*) and, hence, the formation of glucose-1-phosphate would not cause any decrease in acid-labile phosphate.

SUMMARY

1. A *Bacillus* species, strain W-2, possesses a glucokinase as a " constitutive " enzyme, which can be extracted from cells by grinding with glass powder.

2. Cell-free preparations prepared and partially purified from the organism phosphorylate glucose with the aid of ATP, but are completely inactive towards fructose. Mannose, galactose and L-arabinose are also phosphorylated by the preparations at rates of 10, 8 and 15 per cent, respectively, of that for glucose, whereas D-glucosamine, D-ribose, D-xylose and sedoheptulose are all inactive.

3. The pH-activity curve of this enzyme has two maxima at pH 6.9 and 8.4.

4. The activity of this enzyme depends on the presence of either Mg^{++} or Mn^{++}. Mg^{++} is twice more effective as activator than Mn^{++} as compared at their respective optimal concentrations.

5. The enzyme is strongly inhibited by Cu^{++}, Hg^{++}, iodoactate and *p*-chloromercuribenzoate. Alloxan is also inhibitory to the enzyme. The inhibition caused by alloxan can be reversed by cysteine.

REFERENCES

(*1*) Sato, R., Ebata, M., and Takemori, S., Abstracts of the 9th Annual Meeting of the Chemical Society of Japan, 1956. p. 182 (in Japanese)
(*2*) Kunitz, M., and MacDonald, M., *J. Gen. Phyiol.*, **29**, 393 (1946)
(*3*) Slein, M. W., Cori, G. T., and Cori, C. F., *J. Biol. Chem.*, **186**, 763 (1950)
(*4*) Brown, D. H., *Biochem. Biophys. Acta*, **7**, 487 (1951)
(*5*) Harpur, R. P., and Quastel, J. H., *Nature*, **164**, 693 (1949)
(*6*) Cori, G. T., Ochoa, S. Slein, M. W., and Cori, C. F., *Biochem. Biophys. Acta*, **7**, 304 (1951)
(*7*) Slein, M. W., Cori, G. T., and Cori, C. F., *J. Biol. Chem.*, **186**, 763 (1950)
(*8*) Leuthardt, F., and Testa, E., *Helv. Chim. Acta*, **33**, 1919 (1950)

(9) Leuthardt, F., and Testa, E., *Helv. Chem. Acta*, **34**, 931 (1951)
(10) Klein, H. P., and Doudoroff, M. *J. Bact.*, **59**, 739 (1950)
(11) Cardini, C. E., *Enzymologia*, **14**, 362 (1951)
(12) Sato, R., Ebata, M., and Kojima T., *J. Biochem.*, **41**, 307 (1954)
(13) Ebata, M., Sato, R., and Bak, T., *J. Biochem.*, **42**, 715 (1955)
(14) Sumner, J. B., *J. Biol. Chem.*, **65** 393 (1925)
(15) Dixon, M., and Webb, E. C., *Brit. Med. Bull.*, **9**, 110 (1953)
(16) Griffiths, M., *Arch. Biochem.*, **20**, 451 (1949)
(17) Klein, H. P., *J. Bact.*, **66**, 650 (1953)
(18) Ebata, M., Morita, J., and Sato, R., *J. Jap. Biochem. Soc.*, **28**, 4 (1956) (in Japanese)
(19) Axelrod, B., Saltman, P., Bandurski, R. S., and Baker, R. S., *J. Biol. Chem.*, **197**, 89 (1952)
(20) Saltman, P., *J. Biol. Chem.*, **200**, 145 (1953)
(21) Umbreit, W. W., Burris, R. H., and Stanffer, J. F., " Manometric techniques and tissue metabolism," Revised ed., Burgess Pub. Co., p. 199, Minneapolis, (1949)

The Journal of Biochemistry, Vol. 43, No. 5, 1956

SIMPLIFIED LUMIFLAVIN METHOD FOR THE MICRO-DETERMINATION OF FLAVIN COMPOUNDS IN ANIMAL TISSUES

By KUNIO YAGI

(*From the Department of Biochemistry, School of Medicine, Nagoya University, Nagoya*)

(Received for publication, May 9, 1956)

For the micro-determination of flavin compounds in animal tissues, Bessey *et al.* (*1*) had already reported a method suitable for the separative determination of flavin compounds. In determination of the total quantity of flavin compounds by this method, however, it requirs 24 hours for the hydrolysis of flavin-adenine dinucleotide (FAD) to flavin mononucleotide (FMN) by trichloroacetic acid.

To estimate the total quantity of flavin compound in short time, the lumiflavin fluorescence method is more convenient, for this method gives the result in 1 or 2 hours. When the respective quantities of flavin compounds are to be determined, the ratio of each other obtained from the warm-water extract (*2*) by using filter paper chromatography (*3*) or paper electrophoresis (*4*), or by separating each other by ion-exchange resins (*5*) can be adopted.

Accordingly, the lumiflavin fluorescence method, if simple and exact, is considered to be excellent one for the micro-determination of flavin compounds. Though the lumiflavin method is not quantitative for the flavin solution of relatively high concentration (*6*), it was proved to be practiced quantitatively in dilute flavin solutions extracted from bacteria (*7*).

Using a micro-photofluorometer, the micro-determination procedure was examined in detail. The results and the standard procedure are described in this paper.

MATERIALS AND APPARATUS

Flavins—Free riboflavin (FR) and FMN were furnished from Hoffman-La Roche Co. FMN was purified by electrophoresis on a thick filter paper to remove a small amount of FR and other derivatives (*5*).

FAD was the powder extracted from *Eremothecium Ashbyii* (*8*). The purity of

it was over 85 per cent, and it contained neither nucleic acid nor other flavin compounds.

Glass-stoppered Centrifuge Tube—For the irradiation of these flavins to convert to lumiflavin, glass-stoppered centrifuge tubes, as shown in Fig. 1, were used. Using these tubes, the procedures following irradiation can be conducted without changing tubes.

Micro-photofluorometer—To estimate the fluorescence of lumiflavin, a micro-photofluorometer designed by Yagi and Arakawa (*9*, *10*) was employed. Using this apparatus, instructions of meter were parallel with the concentrations of lumiflavin in chloroform solution within the range of 1.0×10^{-4}—2.5 γ/ml. (*10*).

FIG. 1. Glass-stoppered centrifuge tube for photodecomposition and chloroform extraction procedure.

EXPERIMENTAL

Condition of Photodecomposition

As the photodecomposition of flavins to lumiflavin is the chief procedure of the method, the conditions were examined in detail.

Light Source and Irradiation Time—It was already demonstrated that visual light of shorter wave length than 530 mμ, other than ultraviolet ray, is also effective for the photodecomposition of flavins (*11*), and that fluorescent lamp (Mazda FL 20 D) is more excellent than ordinary tungsten lamp or high pressure mercury lamp as a practical light source for the photodecomposition of flavins (*12*). So, an apparatus shown in Fig. 2, was used for the photodecomposition.

To know suitable time for iradiation by this apparatus, the following experiments were carried out. Each 2.0 ml. of the aqueous solution of 0.5 γ/ml. (1.33×10^{-9} mol./ml.) of riboflavin or equimolar concentration of FMN or FAD was mixed with equal volumes of aqueous N NaOH, irradiated for varying periods, acidified with 0.2 ml. of acetic acid, and then lumiflavin produced from flavins was extracted with 6.0 ml. of chloroform. The intensity of the florescence of chloroform layer was measured by the micro-photofluorometer, and the results are shown in Fig. 3. It can be seen that the suitable time of irradiation under these conditions lies in 30–60 minutes.

Temperature of Solution—Each 2.0 ml. of the above-mentioned aqueous flavin solution was mixed with equal volume of N NaOH, irradiated

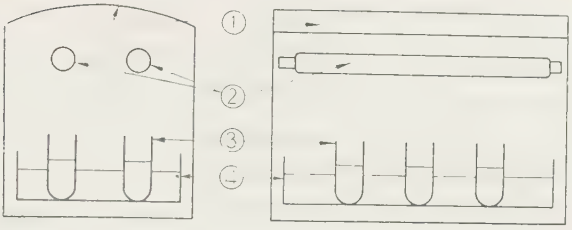


FIG. 2. Apparatus for irradiation to convert flavins to lumiflavin.

1, Mirror; 2, Fluorescent lamp (Mazda FL 20 D); 3, Glass-stoppered centifuge tube without stoppers; 4, Holder for centrifuge tubes.

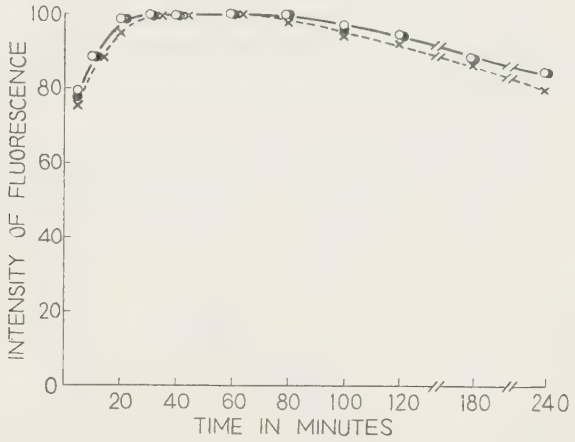


FIG. 3. Rate of lumiflavin obtained in each time of irradiation. ○—○, FR; ●—●, FMN; ×—×, FAD.

for 30 minutes at different temperatures of the solution. The results are shown in Fig. 4, which indicates that 20° is the most suitable temperature for each flavin solutions, and that within 10–30° the rate of lumiflavin production is almost same in three flavin derivatives.

pH of Solution—Each 2.0 ml. of the above-mentioned aqueous flavin solution of was mixed with equal volume of *M*/10 glycine-NaOH buffer. In each tube, final concentration of glycine was adjusted to *M*/20, and pHs values of the solutions were graduated from 7 to 14. After irradiating at 20° for 30 minutes, lumiflavin produced was extracted with chloroform

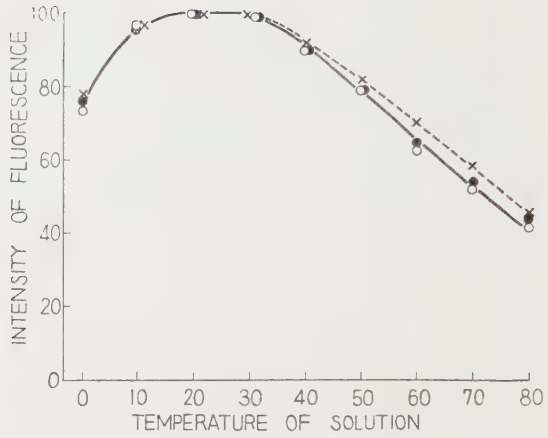


Fig. 4. Effect of temperature on the rate of lumiflavin obtained.
○—○, FR; ●—●, FMN; ×—×, FAD.

and the intensity of the fluorescence of it was measured. As shown in Fig. 5, it indicates that pH of the solution must be strongly alkaline above pH 13 to obtain the same rate of lumiflavin production in each of the three flavins.

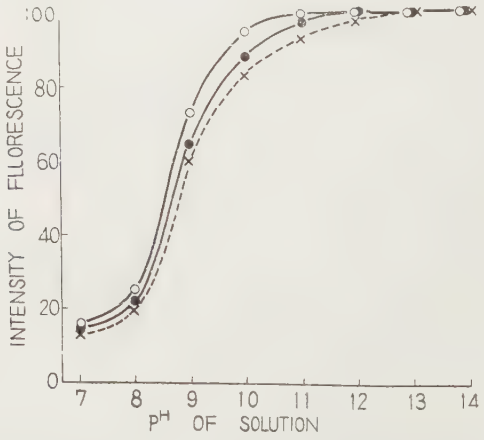


Fig. 5. Effect of pH on the rate of lumiflavin obtained.
○—○, FR; ●—●, FMN; ×—×, FAD.

Relation between Flavin Quantity and Fluorometer Reading

In the Case of Flavin Aqueous Solution—Two ml. of he above-mentioned aqueous flavin solution was mixed with equal volume of *N* NaOH, irradiated under the best condition obtained from the above experiments, and was extracted three times with each 5 ml. of chloroform. The chloroform layers were combined and the intensity of fluorescence was estimated. By comparing with the intensity of fluorescence of standard lumiflavin in chloroform solution, the production of lumiflavin was calculated as 95 per cent.

As a practical method to estimate flavin quantity, a single chloroform extraction for once will be sufficient to evalute the initial flavin quantity, unless the volumes of the irradiated solution and chloroform layer are not changed in each tube. Using this procedure, the relation between flavin quantity and fluorometer reading was examined by the following experiments. Each 2.0 ml. of graduated concentration of flavin aqueous solution was mixed with equal volume of *N* NaOH, irradiated for 30 minutes at 20°, acidified with acetic acid, and then lumiflavin was extracted with 6.0 ml. of chloroform. The intensity of the fluorescence of chloroform layer was estimated. The results are shown in Fig. 6.

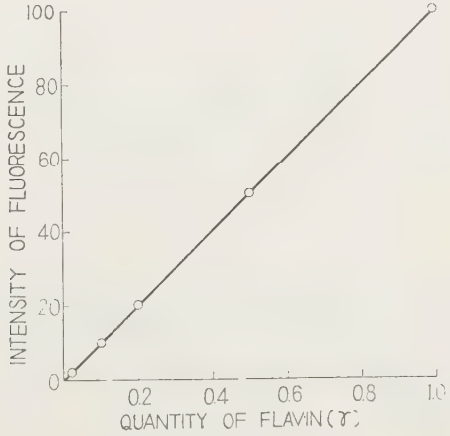


Fig. 6. Relation between initial quantity of flavin and the intensity of fluorescence of chloroform layer.

The quantities of FMN and FAD were calculated as FR. The results with FR, FMN and FAD were agreed on the same points of the figure.

From the results, it is considered that intensity of fluorescence exactly shows the initial quantity of flavin.

In the Case of Flavins in Animal Tissues—The warm-water extract (*2*) of the liver of hen (0.5 g./20 ml.) was used as a sample. Each graduated volume of the solution was made up to 2.0 ml. with water and operated in the same way as described above.

As shown in Fig. 7, fluorometer reading exactly shows the quantity taken. Using the extract of the kidney or the intestine, the same results were obtained.

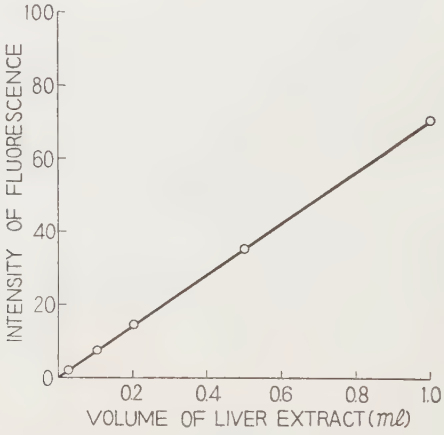


Fig. 7. Relation between the volume of the liver extract taken and the intensity of the fluorescence of chloroform layer.

Addition Test

Each 0.5 ml. of water extract of hen liver (0.1 g./20 ml.) was put into centrifuge tube. To these tubes, aqueous FR solutions were added and made up to 2.0 ml. so as to contain graduated quantity of flavins. These tubes were operated in the same way as described above, and the results are shown in Fig. 8. From the results, it is clear that fluorometer reading exactly shows the flavin quantity added.

Standard Procedure

As a method for extracting flavins from animal tissues, "warm-water-extraction" method (*2*) was found to be the most convenient one.

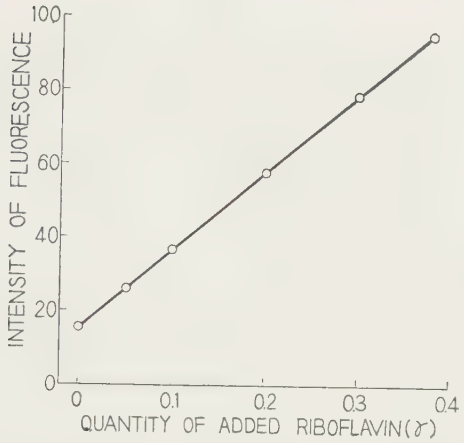


FIG. 8. Relation between the quantity of added FR and the intensity of the fluorescence of chloroform layer.

So, after the preparation of the extract by this method, the extract is treated by the procedure examined in the above-mentioned experiments. A standard procedure is given as follows:

Extraction with warm water—Tissue is excised fresh from animals, immediately weighed (*a* g.), and cut into small pieces. (in ordinary cases, 0.1–1.0 g. of tissue is used). A few ml. of water warmed at 80° are added, and warmed at 80° for 3–5 minutes. After grinding it in a glass-homogenizer, the homogenate is transferred into graduated tube, diluted with water up to *b* ml., and warmed at 80° for 15 minutes. After it is cooled at room temperature, the total volume of the extract is made up to *b* ml. exactly with water, stirred, centrifuged, and the supernatant is used as test solution.

Photodecomposition—One ml. of the test solution, 1.0 ml. of water, and 2.0 ml. of aqueous *N* NaOH are placed together in a glass-stoppered centrifuge tube *A*. At the same time, 1.0 ml. of the test solution, 1.0 ml. of 0.1 γ/ml. of FR aqueous solution, and 2.0 ml. of aqueous *N* NaOH are placed together in the tube *B*. (The quantity of FR added must be changed so as to be similar quantity with that of test solution. It will be determined by preliminary experiment.)

Then, tubes *A* and *B* are set in the light irradiation apparatus for 30–60 minutes at 10–30°.

Extraction with Chloroform—To the above two tubes, each 0.2 ml.

of glacial acetic acid and 6.0 ml. of water-saturated chloroform are added, respectively. After they are cooled with cold water, shaken for about 30 seconds, centrifuged, and each 5.0 ml. of chloroform layer of A and B are transferred into cuvettes A' and B', respectively.

On the other hand, 1.0 ml. of test solution, 1.0 ml. of water, 0.2 ml. of glacial acetic acid, and 2.0 ml. of N NaOH are mixed together in the tube C, and extracted with 6.0 ml. of chloroform in the same manner. Five ml. of chloroform layer are put into cuverte C'.

Estimation of Fluorescence—The dark electric current of micro-photofluorometer is regulated at 0. After cuvette B' is set in the light path, shutter is opened, and then the slit of micro-photofluorometer is opened gradually until the compass needle notices near 100. The reading is noted as f_1, then A' and C' are estimated under the same condition, and the reading are noted as f_2 and f_3 respectively.

Calculation—The difference of f_2-f_3 corresponds to the flavin content of the test solution, and f_1-f_2 to that of FR added.

So, the quantity of flavins calculated as FR in animal tissue, in the case of 0.1 γ of FR was added, is

$$0.1\times\frac{f_2-f_3}{f_1-f_2}\times\frac{b}{a}\quad \gamma/\text{g.}$$

Example of Measurement

Using the above-mentioned standard procedure of this method, flavin contents of several animal tissues were estimated, and the results are shown in Table I.

TABLE I

Amount of Flavin in Animal Tissues

		γ/g.			γ/g.
White rat	Liver	33.4	White rat	Brain	3.2
	Kidney	33.1		Muscle	3.0
	Heart	18.8		Liver	31.2
	Stomach	6.6		Kidney	30.5
	Intestine	5.1	Rabbit	Heart	18.0
	Spleen	5.0		Spleen	6.3
	Testicle	3.7		Lung	3.1

The values are calculated as free riboflavin on the basis of wet tissue weight.

DISCUSSION

For the determination of flavins in living tissues, the fluorometric method is suitable, because flavin content of living body is generally small. To estimate the fluorescence of flavin separately from similar fluorescent substances in living body, the procedure of converting flavins to lumiflavin is of excellent one. In animal tissues, the fluorescent substances soluble in chloroform is in neglisible amount. So, the procedure described above is reliable to determine flavins in tissues.

If a sample contains a certain amount of fluorescent substances soluble in chloroform, these substances can be removed by extracting with chloroform before photodecomposition.

When similar samples are determined repeatedly, the procedure can be more simplified by using standard curve of addition test.

By the above mentioned standard procedure, several samples can be estimated in 2 hours.

Further, when separative determination of flavin nucleotides is required, the procedure using filter paper chromatography (*3*), or filter paper electrophoresis (*5*) can be used satisfactorily by combining with this method, and also the ion-exchange separation (*4*) will be applicable.

SUMMARY

To estimate flavin compounds in animal tissues by lumiflavin fluorescence method, the conditions of photodecomposition of flavins to lumiflavin were examined in detail using dilute flavin solutions. The best condition for photodecomposition was 30–60 minutes irradiation by fluorescent lamp at 10–30° of solution temperature and solution pH higher than 13. Under these conditions, the amount of lumiflavin produced exactly showed the original flavin quantity.

A standard procedure is as follows: Warm-water extract of tissue is mixed with equal volume of *N* NaOH, irradiated under the above conditions, extracted once with chloroform and intensity of fluorescence of chloroform layer is estimated. At the same time, the addition test is made by the same way, and amount of flavin is calculated.

By this procedure, flavin quantities of several samples will be estimated simply within 2 hours.

The author wishes to thank Mr. Jun Okuda for his valuable technical assistance

in the course of this work.

REFERENCES

(1) Bessey, O. A., Lowry, O. H., and Love, L. H., *J. Biol. Chem.*, **180**, 755 (1949)
(2) Yagi, K., and Ishiguro, I., *Medicine and Biology* (Japan), **17**, 105 (1950)
(3) Yagi, K., *J. Biochem.*, **38**, 161 (1951)
(4) Yagi, K., and Matsuoka, Y., *J. Biochem.*, **42**, 757 (1955)
(5) Yagi, K., Okuda, J., and Matsuoka, Y., *Nature*, **175**, 555 (1955)
(6) Kuhn, R., Wagner-Jauregg, T., and Kaltschmitt, H., *Ber. dtsch. chem. Ges.*, **67**, 1452 (1934)
(7) Yagi, K., and Mitsuhashi, S., *Jap. J. Exp. Med.*, **21**, 353 (1951)
(8) Yagi, K., Matsuoka, Y., Kuyama, S., and Tada, M., *J. Biochem.*, **43**, 93 (1956)
(9) Yagi, K., and Arakawa, T., *Science* (*Japan*), **23**, 216 (1953)
(10) Yagi, K., and Arakawa, T., *Vitamins* (*Japan*), **6**, 523 (1953)
(11) Yagi, K., *Medicine and Biology* (*Japan*), **28**, 54 (1953)
(12) Yagi, K., *Vitamins* (*Japan*), **7**, 493 (1954)

The Journal of Biochemistry, Vol. 43, No. 5, 1956

HEXOKINASES OF RABBIT TESTIS

By IKURO AKAEDA

(From the Department of Biochemistry, Okayama University Medical School, Okayama)

(Received for publication, May 16, 1956)

Hexokinases which phosphorylate carbon 1 in the presence of ATP and Mg^{++} have been described for fructose and galactose. However, no enzyme of this type has been reported for glucose. Recently, hexokinases of rabbit testis were investigated in the normal and diabetic states, and it was found that rabbit testis appeared to have C-1-glucokinase which was inhibited in the diabetic state induced by alloxan. Testis fructokinase which phosphorylates carbon 6 was not affected in the diabetic state.

METHODS

Diabetic rabbits were provided by intravenons injection of alloxan (200 mg. per kg. body weight).

0.8 ml. of 33 per cent isotonic KCl homogenates of testis from normal or alloxan diabetic rabbits were mixed with 0.2 ml. of 0.056 *M* (1 per cent) glucose or fructose, 0.2 ml. of 0.38 *M* $NaHCO_3$, 0.2 ml. of 0.06 *M* ATP, 0.2 ml. of 0.8 *M* $MgCl_2$ and 0.2 ml. of 0.5 *M* NaF. The mixture was adjusted to pH 7, and the final volume was made up to 2.0 ml.

One of the two tubes of the above mixture was analysed at once without incubation for the amount of free glucose or fructose and acid labile phosphate. Another tube was incubated for twenty minutes at 37° in a constant temperature bath. Nitrogen was used as a gas phase. Before incubation, all the operations were carried out at O°. Both mixtures, before and after incubation, were deproteinized by addition of 10 ml. of perchloric acid and neutralized with 5 *N* KOH. The neutral solutions were filled up to 20 ml. with distilled water, and filtered. 2 ml. of the filtrate was fixed with each of 4 ml. of $ZnSO_4$ and $Ba(OH)_2$. 2 ml. of Zn-Ba filtrate were analysed for free glucose by Nelson's method (*1*) and for fructose by Roe's method (*2*). For the determination of acid labile phosphate, 3 ml. of the filtrate from the neutralized solution was mixed with 0.3 ml. of 11 *N* HCl and the mixture was heated for seven minutes at 100° in a boiling water bath. After cooling, one ml. of the mixture was used for the color development (*3*). Optical densities were measured by a photoelectric colorimeter (Shimazu Ltd.).

RESULTS

Testis Glucokinase from Normal and Diabetic Rabbits

The data is shown in Table I. In the normal rabbits, there was a marked difference between initial and final glucose, *e.g.* glucose was phosphorylated. However, there was no difference between initial and final acid labile phosphate. These facts indicate that phosphate ester of glucose thus produced is glucose-1-phosphate. Experiments were repeated four times.

Testis glucokinase was inhibited in the diabetic state as shown in the Table I.

TABLE I

Glucokinase from Normal and Diabetic Rabbits

Experiment No.		Glucose (μM)			Acid labile phosphate (μM)		
		Initial	Final	Esterified	Initial	Final	Decreased
Normal	1	13.4	9.1	4.4	18.9	18.5	0.4
	2	11.6	6.3	5.3	18.1	17.9	0.2
	3	14.1	8.3	5.8	13.4	13.4	0.0
	4	15.0	6.5	8.5	11.7	12.0	−0.3
Diabetic	1	11.6	10.8	0.8	19.0	19.2	−0.2
	2	11.0	10.5	0.5	17.5	17.5	0.0

Testis Fructokinase from Normal and Diabetic Rabbits

The data is shown in Table II. In the normal testis, fructose was used and the acid labile phosphate was decreased after incubation. This fact indicates that phosphate ester of fructose produced is fructose-6-phosphate. In the diabetic state, the fructokinase was not inhibited.

DISCUSSION

In most of the animal tissues so far tested glucose was phosphorylated at carbon 6 (*4*, *5*). In these experiments, fluoride was used to inhibit phosphatase hydrolyzing phosphate ester produced. Fluoride is also known to inhibit phosphoglucomutase which transforms 95 per cent of glucose-1-phosphate into glucose-6-phosphate. This fluoride

TABLE II

Fructokinase from Normal and Diabetic Rabbits

Experiment No.		Fructose (μM)			Acid labile phosphate (μM)		
		Initial	Final	Esterified	Initial	Final	Decreased
Normal	1	11.0	8.9	2.1	18.3	16.5	2.8
	2	13.2	9.5	3.7	10.9	7.6	3.3
Diabetic		11.8	8.8	3.0	10.1	6.8	3.3

inhibition of phosphoglucomutase is, however, a special case and is dependent on the concentration of three ions, magnesium, fluoride and glucose-1-phosphate. Hence, there remains a possibility to find C-1-glucokinase in some tissues by using arsenate as an inhibitor (*6*). Although arsenate was not used in this experiment, it seems to be quite probable that rabbit testis contains C-1-glucokinase. A definite proof of glucose-1-phosphate produced is now going on. Sugar metabolism of testis may have a different pathway.

SUMMARY

1. Rabbit testis appears to contain C-1-glucokinase which is inhibited in diabetic state.

2. Fructokinase of rabbit testis which phosphorylates carbon 6 is not affected in diabetes.

The author is indebted to Dr. S. Mizuhara for his generous advice. This investigation was supported by the Grant for Scientific Research of the Department of Education.

REFERENCES

(*1*) Nelson, N., *J. Biol. Chem.*, **153**, 375 (1949)
(*2*) Roe, T. H., *J. Biol. Chem.*, **107**, 15 (1934)
(*3*) Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, **66**, 375 (1925)
(*4*) Ooishi, T., *J. Japan Biochem. Soc.*, **27**, 653 (1956)
(*5*) Leloir, L. F., in Phosphorus metabolism (McElroy and Glass, eds.), Vol. **I**, 67, Johns Hopkins Press, Baltimore (1951)
(*6*) Leloir, L. F., and Trucco, R. E., in "Methods in enzymology" (Colowick and Kaplan, eds.), Vol. **I**., 290–294, Academic Press, N. Y. (1955)

The Journal of Biochemistry, Vol. 43, No. 5, 1956

CONFIRMATION OF GLUCOSE-1-PHOSPHATE PRODUCED BY GLUCOKINASE OF RABBIT TESTIS

By IKURO AKAEDA

(*From the Department of Biochemistry, Okayama University Medical School, Okayama*)

(Received for publication, May 16, 1956)

Shortly before, it has been reported that rabbit testis might contain C-1-glucokinase (*1*). This interpretation was deduced by measuring the free glucose used and the difference of acid labile phosphate before and after incubation. Present comunication is concerned with the confirmation of glucose-1-phosphate (G-1-P) produced by the testis glucokinase. A paper chromatographic method (*2*) was used for this purpose.

MATERIALS AND METHODS

Glucose-6-phosphate (G-6-P) and G-1-P used for the identification of the spots on paper were kindly furnished by Dr. P. Handler of Duke University (U.S.A.).

Adenosine triphosphate (ATP) was prepared from rabbit muscle by the method of Dounce (*3*). Incubation mixture had the same constitution as described before (*1*), but its volume was doubled. Nitrogen containing 5 per cent CO_2 was used as a gas phase in Thunberg tubes. Without incubation (control tube) or after twenty minutes incubation (main tube) at 37°, barium soluble-alcohol insoluble fraction was prepared by the usual method (*4*). The sugar phosphate fraction thus obtained was dried with cold absolute alcohol and stored in a desiccator at O°. The dried meterial was dissolved in a minimal amount of 0.1 *N* hydrochloric acid and the barium ion were removed by adding sodium sulfate. All the procedures were carried out at O°. After centrigugation in a refrigerated centrifuge, the supernatant was neutralized and applied on filter papers (Toyo Roshi No. 50). Chromatographic procedure used in this experiments such as solvents and spraying agents were essentially the same as reported by Bandurski and Axelrod (*2*), but Rf values of sugar phosphates obtained in this laboratory were different from those reported by them.

RESULTS

Fig. 1 shows one dimensional chromatogram in an acid solvent (methanol 80 vol., 80 per cent formic acid 16.5 vol., H_2O 3.5 vol.).

Fig. 1 (a) shows the chromatogram of the sugar phosphate fraction from the control tube.

Fig. 1 (b) shows that of the same fraction from the main tube which was incubated for twenty minutes at 37°. Spot No. 1 (Rf:0.41) appeared only after twenty minutes incubation at 37°. When authentic

Fig. 1. One dimensional chromatogram.

The Thunberg tubes contain the following substances in a final volume of 4 ml.:1.6 ml. of 33 per cent isotonic KCl homogenate of rabbit testis, 0.4 ml. of 1 per cent glucose, 0.4 ml. of 0.38 *M* $NaHCO_2$, 0.4 ml. of 0.06 *M* ATP, 0.4 ml. of 0.8 *M* $MgCl_2$, and 0.4 ml. of 0.5 *M* NaF. Main tube was incubated at 37° for 20 min. in 95 per cent N_2-5 per cent CO_2, and control tube was not incubated. Barium soluble-alcohol insoluble fraction was applied on papers.

sample of G-1-P was mixed on a filter paper with the sugar phosphate fraction of the main tube, G-1-P was appeared just in piles on the No. 1 spot (Fig. 1. (c)). The chromatogram of authentic samples of G-1-P, G-6-P and inorganic phosphate was shown in Fig. 1 (d). From these results, spot No. 1 is considered to be G-1-P and spot No. 3 (Rf: 0.52) to be G-6-P. A faint small spot of No. 2 may be considered to be frucotse-6-phosphate (F-6-P) from its Rf value (0.45). The Rf value of inorganic phosphate (spot No. 4) was 0.63–0.64. The Rf values of G-1-P and G-6-P in the basic solvent (methanol 60 vol., NH_4OH 10 vol., H_2O 30 vol.) were 0.66 and 0.65, respectively, thus in this solvent the separate stops of these two sugar phosphates were not obtained in both cases of barium soluble-alcohol insoluble fraction from the main tube and of authentic samples.

DISCUSSION

About the origin of G-6-P observed on both filter papers of contro. and main experiments, the following interpretations are deduced:

(1) G-1-P was contained in the homogenate from the first.

(2) G-6-P was derived from F-6-P by the action of phosphohexo-isomerase which transforms 70 per cent F-6-P into G-6-P. F-6-P will be formed from ATP and fructose which might be contained in the homogenate.

(3) The inhibition of phosphoglucomutase by NaF is not perfect as discussed previously (*1*), so G-1-P exist or formed from glucose in the homogenate will be able to be transformed into G-6-P to a certain extent.

A possibility of the G-1-P formation from testis glycogen by the action of phosphorylase remains to be considered. So an experiment was repeated without added ATP, and it was found that glucose added was never utilized and no spot of G-1-P was detected on a paper chromatogram in an acid solvent.

From these results, it seems quite probable that rabbit testis has contained C-1-glucokinase. Further evidences and the significance of the presence of C-1-glucokinase in rabbit testis will be chased anyway in the comming papers.

SUMMARY

Glucose, ATP, Mg^{++}, and NaF were mixed with the homogenate

of rabbit testis, and the barium soluble-alcohol insoluble fraction of the mixture was examined by paper chromatography. A large spot of G-1-P was detected only after incubation at 37° for twenty minutes. Without incubation, no spot of G-1-P was observed. So it seems quite probable that rabbit testis contains C-1-glucokinase.

The author is indebted to Dr. S. Mizuhara for his continued interest and to Dr. T. Baba for his technical advice.

This investigation was supported by a scientific research grant from the Department of Education.

REFERENCES

(*1*) Akaeda, I., *J. Biochem.*, **43**, 645 (1956)
(*2*) Bandurski, R. S., and Axelrod, B., *J. Biol. Chem.*, **103**, 405 (1951)
(*3*) Dounce, A. L., *J. Biol. Chem.*, **174**, 361 (1948)
(*4*) Umbreit, W. W., Burris, R. H., and Stauffer, J. F., Manometric techniques and tissue metabolism, Burgess Pub. Co., Minneapolis, 2nd edition (1949)

The Journal of Biochemistry, Vol. 43, No. 5, 1956

THE IN VITRO INCORPORATION OF C^{14}-GLYCINE INTO ANTIBODY AND OTHER PROTEIN FRACTIONS BY POPLITEAL LYMPH NODES OF RABBITS FOLLOWING THE LOCAL INJECTION OF CRYSTALLINE OVALBUMIN

By KIKUO OGATA*, MASANA OGATA**, YOSHIO MOCHIZUKI*** and TADAMOTO NISHIYAMA*

(From the Department of Biochemistry, Niigata University School of Medicine, Niigata, and the Department of Public Health** and Hygiene***, Okayama University School of Medicine, Okayama)*

(Received for publication, May 17, 1956)

Since the study of Ehrlich and Harris (*1–4*), it has been generally accepted that the popliteal lymph nodes are the active sites in the formation of antibody. Recently M. Ogata and Y. Mochizuki (*5*) (authors of the present paper) demonstrated, employing the quantitative precipitin method of Heidelberger (*6–8*), that precipitable antibody nitrogen per gram tissue of the popliteal lymph nodes were greater than that of serum in rabbits immunized by the foot-pad injection of ovalbumin, and confirmed the above fact.

On the other hand, Heidelberger *et al.* (*9*) demonstrated that the N^{15}-glycine was incorporated *in vivo* into the newly formed antibody protein.

However, there have been only few reports concerning the formation of antibody in the tissue preparation *in vitro*. Roberts *et al.* (*10*) demonstrated that minced lymphoid tissue from rats and mice immunized to sheep erythrocytes, released antibodies during the incubation with suitable medium and suggested that antibody production might occur *in vitro*. Recently, Ranney and London (*11*) reported briefly that slices of the liver and spleen from rabbits immunized with type III pneumococci incorporated C^{14}-glycine into antibody, and Askonas and Humphrey (*12*) reported similar results using the slices of granulomata produced in rabbits by injection of Freud's adjuvant containing ovalbumin.

But there have been neither reports on the rate of the incorporation of C^{14}-amino acid into antibody and other protein fractions of the immunized tissue preparation, nor reports concerning various metabolic

factors governing the rate of the incorporation.

For these reasons, the authors have undertaken a fundamental study in which the rate of incorporation of C^{14}-glycine into antibody and other protein fractions were determined and further various metabolic factors affecting the incorporation rate were investigated in detail, using cell suspensions or homogenates of popliteal lymph nodes of rabbits afte the local immunization with crystalline ovalbumin.

EXPERIMENTAL

Treatment of Animals—The rabbits used for immunization were albino ranging in weight from 2 to 3 kg. They were given the subcutaneous injection of alum-precipitated crystalline ovalbumin in 8 mg. dose at foot pads every other day at least for 4 weeks. Thereafter the popliteal lymph nodes from two or three animals were removed and used for each isotopic experiment. The ovalumin was prepared by the method of Kekwick (*30*).

Tissue Preparation—The following manipulation was performed in the cold room ($-2°$ to $+2°$). To obtain cell suspensions, two kinds of procedures were employed, the one, in which the popliteal lymph nodes were grinded with a mortar and pestle, then passed through a tissue masher fitted with a screen containing holes 0.5 mm. in size (cell suspension-1), the other, in which the lymphatic tissues were cut into very small pieces (0.5–1 mm. diameter or more) with curved ophthalmic scissors (cell suspension-2). They were transferred into a conical centrifuge tube with the aid of the modified Krebs-Ringer-Phosphate (m-K.R.P.) solution described below. After centrifugation the packed cells were washed twice with m-K.R.P. solution and diluted to the appropriate volume with the same solution. The aliquot of this suspension (usually 1 ml.) were added to each main compartment of the Warburg vessel.

To obtain cell free homogenate the following method was employed, in which the tissue was grinded with a mortar and pestle after the addition of small amounts of powdered glass and homogenization medium described bellow. The homogenate was centrifuged at 1500 r.p.m. for five minutes, then the supernatant was used for the experiment.

Reaction Mixture—Two kinds of modified Krebs-Ringer-Phosphate solutions were used as suspending media of the tissue slices. The one (m-K.R.P.-1) was prepared by mixing stock solutions of 110 ml. of 0.9 per cent NaCl, 4 ml. of 1.15 per cent KCl, 1 ml. of 3.82 per cent $MgSO_4$ 7 aq. and 6 ml. of 0.1 *M* sodium phosphate buffer (pH 7.8). The other (m-K.R.P.-2) was prepared by mixing stock solutions of 100 ml. 0.9 per cent NaCl, 1 ml of 3.82 per cent $MgSO_4$7 aq., 4 ml. of 1.15 per cent KCl, 6 ml. of 0.1 *M* sodium phosphate buffer (pH 7.8) and 3 ml. of 1.22 per cent $CaCl_2$, which was added immediately before the experiments.

Composition of Homogenization Medium—The usual constituents were as follows: KCl 0.120 *M*, potassium phosphate buffer (pH 7.4) 0.04 *M*, and $MgCl_2$ 0.004 *M*.

C^{14}-Glycine—Three kinds of 1-C^{14}-glycine were used in the experiment, their speci-

fic activities being 8.5, 19.3 and 38.5 μC. per mg., respectively.

Incubation Procedure—The lymphatic cell suspensions were incubated with C^{14}-glycine in the main compartments of the Warburg vessels. Each of their center cups contained 0.2 ml. of 10 per cent KOH and a folded filter paper. The gas phase was air or oxygen. The incubation was carried out by shaking at 37°, and the stopcocks were closed after 10 minutes equilibration period. For the short time experiment C^{14}-glycine was added in the reaction mixture from the side arm of the vessel after the equilibration period. At the end of the incubation, the flask content was poured into a ice cold tubes of Potter and Elvehjem's glass homogenizer (*13*), washed twice with 1 ml. of 0.9 per cent NaCl solution and homogenized in the cold room, transferred into a centrifuge tube, lyophilized twice, and then the separation of antibody and the fractionation of other tissue proteins were carried out as follows.

Separation of Antibody and Fractionation of Tissue Proteins—For the separation of antibody, Heidelberger's quantitative precipitin method (*6–8*) was employed and the quantity of precipitated nitrogen was determined by Folin's tyrosine method (*14*). The fractionation of tissue protein was carried out employing the method of Griffin (*15*). The detailed procedure was as follow: after the extraction of soluble protein and ribonucleoprotein by lyophilizing the cells, the extract was centrifuged for 15 minutes at 20,000 r.p.m. in the No.40 Rotor of the Spinco Ultracentrifuge. The precipitate which contained desoxyribonucleoprotein was fractionated as described below.

The supernatant was taken into a centrifuge tube, adjusted to pH 4.8–5.0 by adding 0.2 *N* acetic acid and centrifuged. The precipitate was ribonucleoprotein fraction and washed once with 0.4 *M* NaCl. An appropriate amount of egg albumin, which was determined previously by Heidelberger's method, was added to the supernatant, incubated for 15 minutes at 37°, then placed in the refrigerator overnight. After the precipitation was complete, the centrifugation was carried out at 3000 r.p.m. in the cold room. The specific precipitate (antigen-antibody complex) was washed twice with ice cold 0.9 per cent NaCl solution. The residual supernatant was designated as soluble protein fraction.

The precipitate, which contained desoxyribonucleoprotein, was ground completely with a glass rod and extracted with 2 ml. of 1 *M* NaCl with stirring, and centrifuged for 15 minutes at 20,000 r.p.m. To the supernatant, 1.5 volume of cold distilled water was added and centrifuged at 20.000 r.p.m. for 10 minutes. The precipitate thus prepared was desoxyribonucleoprotein fraction, which was obtained as typical gel form.

Fractionation of cellular components of the lymphatic tissue was carried out by the Schneider centrifugal fractionation procedure using an isotonic sucrose solution (*26*). From nuclear fraction, desoxyribonucleoprotein was fractionated by the procedure as described above.

Washing, Plating and Counting the Protein—Antibody (antigen-antibody complex) and each protein fraction were precipitated with trichloroacetic acid (TCA) (final concentration, 10 per cent), washed three times successively with 5 ml.-portion of 5 per cent TCA with centrifugation. Thereafter each precipitate was washed with

cold 95 per cent ethanol, twice with hot ethanol-ether, then dispersed in 3 ml. of 5 per cent TCA and heated for 15 minutes at 90° to remove nucleic acid. The residual precipitate was washed twice with 5 per cent TCA and then ethanol. The residual pelletes of protein were homogenized in a petroleum ether-ether-acetone solution (6:3:½). This suspension was poured onto stainless steel disc and evaporated to dryness under the infra red rays. After the dried protein was equilibrated in air at least for 1 hour, the weight of the protein on the disc was determined and its radioactivity was estimated either with a thin mica end-window Geiger-Müller Counter or with a Lauritsen Electroscope. All samples were counted long enough to reduce the counting error. The specific activity of the protein[1] was corrected for self-adsorption from an empirically determined curve.

The incorporation data were expressed as μM of glycine per g. protein or μg. C^{14} per g. protein, which was calculated according to Farber, Kit and Greenberg (*16*).

Analytical Method—Lactic acid was determined by the method of Barker and Summerson (*17*).

RESULTS

Experiments with Cell Suspension—As a preliminary experiment, the cell suspensions-1 were incubated with C^{14}-glycine in the m-K.R.P.-1 solution containing glucose in a gas phase of air at 37°. The time course of the incorporation of glycine into antibody and soluble protein was presented in Fig. 1. As shown in this figure, the incoporation proceeded for 2 hours and the rate of uptake of glycine into antibody was greater than that into the soluble-protein, but the differences were not so prominent. Next, under the same experimental conditions, the rate of incorporation of glycine into antibody and the proteins of cellular components, fractionated by Schneider's procedure (*26*), were compared with each other at different time intervals up to 2 hours. As shown in Fig. 2, the glycine uptake into antibody was the most rapid, followed by microsome and soluble proteins, and that into nucleus (desoxyribonucleoprotein) was the slowest.

The effect of the addition of various metabolic inhibitors on the glycine uptake was presented in Table I. It was observed that azide ($5\times 10^{-3}\ M$), arsenate ($10^{-3}\ M$), monoiodoacetate ($2\times 10^{-3}\ M$) and 2.4-dinitrophenol ($5\times 10^{-4}\ M$) inhibited markedly the incorporation of glycine into antibody as well as that into each cellular protein.

1) The specific activity of antibody may be about 10 per cent higher than the value presented in this paper, since in the antigen-antibody precipitate the ratio of the two proteins was about 1:10).

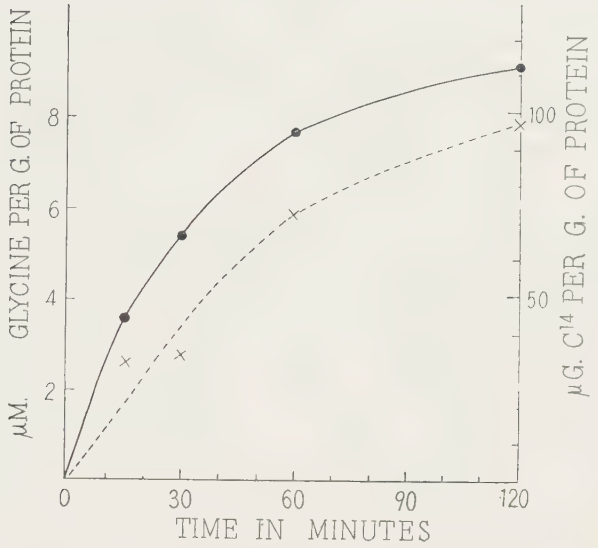


Fig. 1. Rate of the incorporation of glycine into antibody and soluble protein by cell suspensions of the immunized lymph nodes.

●, represents antibody; ×, soluble protein.

Incubation mixture; 1.6 ml. of lymphatic cell suspensions-1 (contained 300 mg. tissue), 0.2 ml. of 0.25 *M* glucose, 0.1 ml. of C^{14}-glycine in 0.9 per cent NaCl. The concentration of glycine (specific activity, 38.5 μC./mg.) was 0.66 m*M*.

Incubation at 37°, in air.

But because under the experimental conditions described above the oxygen uptake by the cellular suspensions was rather low, examinations to arrive at suitable conditions of the cellular respiration were carried out. The best conditions which could be attained, were to use cell suspensions-2 and oxygen as gas phase. In this case, the m-K.R.P.-2 was used as a suspension medium. The results was presented in Fig. 3. The oxygen and amino acid uptake proceeded almost constantly for 2 hours. Especially the incorporation of glycine into antibody increased linearly after a short lag period, which was not observed in other protein fractions. It was remarkable that the specific activity of antibody was much higher than those of the other protein fractions, in which that of the soluble protein showed the next higher value and that

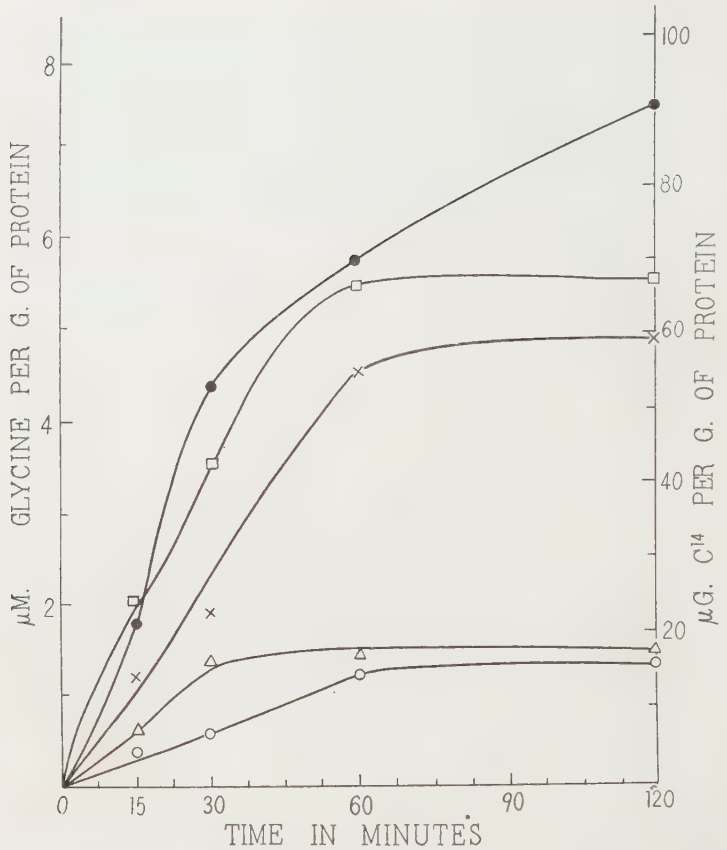


FIG. 2. Rate of the incorporation of glycine into antibody and the proteins of the various cellular fractions by cell suspensions of the immunized lymph nodes.

●, represents antibody □, microsome, ×, soluble protein, △, mitochondria, ○, nucleus. The experimental conditions were the same as those in Fig. 1. except that the concentration of glycine (specific activity, 8.5 μC./mg.) was 2.7 *M*.

Incubation at 37°, in air.

of the protein moiety of the desoxyribonucleoprotein was the lowest. Thus it was indicated that the oxygen uptake by the cell suspension was related closely to the glycine uptake into antibody. The rate of in-

TABLE I(1)

Effects of Various Metablic Inhibitors on the Incorporation by Cell Suspensions of the Immunized Lymph Nodes

(Incorporation expressed as per cent of that without inhibitor)

Experimental No.	Protein fraction				
	No. 8			No. 9	
Addition	Sol-P+ Antibody	RN-P	DN-P	Sol-P	Antibody
None	100 (70§)	100 (65§)	100 (13§)	100	100
Arsenate (10^{-3} *M*)	11	12	(0)†	40	18
Azide (5×10^{-3} *M*)	6	10	40	22	11
2,4-Dinitrophenol (5×10^{-4}*M*)	18	21	(0)†	33	22
Monoiodoacetate (2×10^{-3}*M*)	17	(0)†	(0)†	21	17

TABLE I(2)

Addition	Protein fraction				
	Anti-body	Sol-P*	DN-P*	Mitochon-dria protein	Microsome protein
None	100 (90§)	100 (58§)	100 (16§)	100 (17§)	100 (67§)
Arsenate (10^{-3} *M*)	12	3	12	39	19
Azide (5×10^{-3} *M*)	12	13	8	12	8
2.4-Dinitrophenol (5×10^{-4} *M*)	24	18	23	47	22
Monoiodoacetate (2×10^{-3} *M*)	14	15	30	31	22

The experimental conditions were the same as those in Fig. 2.

§ The figure in parenthesis represents the value of the incorporation expressed μg. C^{14} per g. protein per 2 hours.

† represents the negligible value.

* Sol-P, soluble protein RN-P, protein moiety of ribonucleoprotein, DN-P, protein moiety of desoxyribonucleoprotein.

corporation of C^{14}-glycine, expressed as μM of glycine per gm. protein per hour was represented in Table II. Further the effect of glycine concentration on the incorporation rate was examined, and the results was shown in Table III.

The effect of anaerobiosis was then examined, which was represented

in Table IV. From this Table it can be seen anaerobiosis inhibits almost completely the glycine uptake into antibody as well as those into other protein fractions. The addition of glucose or/and ATP, phosphocreatine and hexosediphosphate in the anaerobic condition increased the lactic acid formation by cell suspensions, while restored only slightly the glycine uptake.

Experiments with Tissue Homogenate—When cell free homogenate was used, the C^{14}-glycine uptake into antibody as well as each protein fraction was greatly reduced, as compared with the value obtained by using intact cell suspensions.

But, as shown in Fig. 4, even in this case the rate of the incorporation of C^{14}-glycine into antibody was observed to be much higher than those of the other protein fractions. The azide (10^{-3} *M*), monoiodoacetate (10^{-3} *M*), 2.4-dinitrophenol (10^{-3} *M*) or malonate (1.2×10^{-3} *M*) inhibited the glycine uptake as shown in Table V.

Fig. 3 (1)

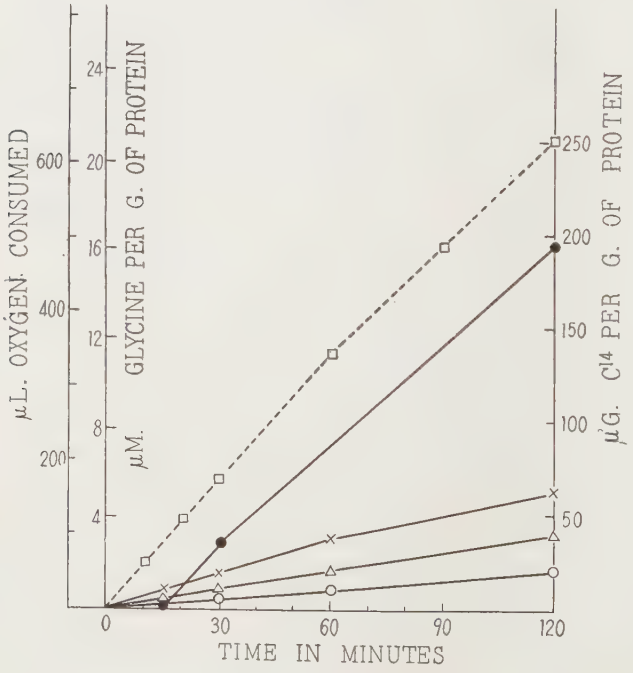


FIG. 3 (2)

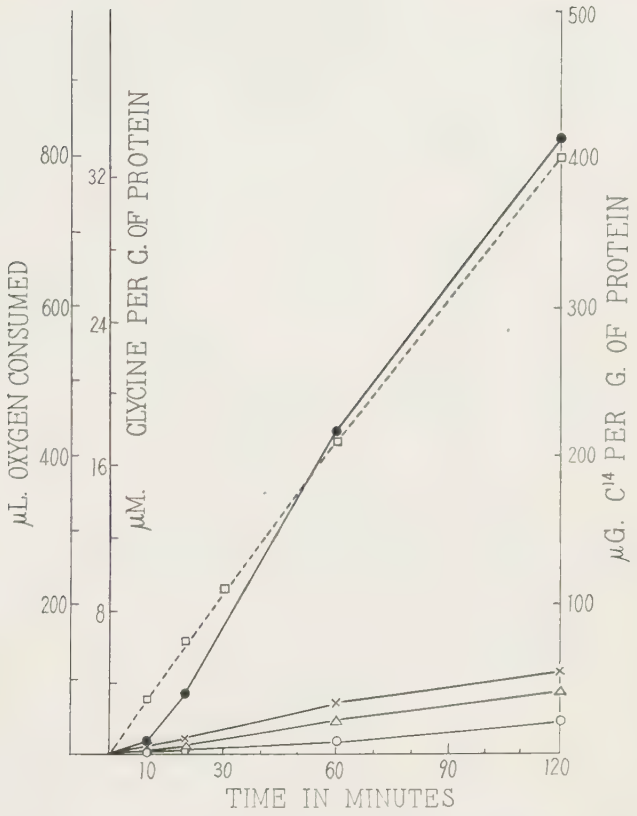


FIG. 3. Rate of oxygen uptake and the incorporation of glycine into antibody and other tissue proteins by cell suspensions-2 of immunized lymph nodes.

...□...□... represents the oxygen uptake, —●—●—, antibody; —×—×—, soluble protein; —△—△—, protein moiety of ribonucleoprotein; —○—○—, that of desoxyribonucleoprotein. The incubation mixture: 1.2 ml. of the cell suspension (containing 500 mg. itssue), 0.4 ml. of 0.9 per cent NaCl and 0.1 ml. of C^{14}-glycine (specific activity, 19.3 μC./mg.). The concentration of glycine was 0.68 m*M*. Incubation at 37°, in oxygen.

TABLE II

Rate of the Incorporation of C^{14}-Glycine into Antibody and Other Cellular Proteins

Experimental No.	Glycine in μM per g. protein per hour			
	Antibody	Sol-P*	RN-P*	DN-P*
1	8.4	3.7	2.1	1.1
2	10.0	2.9	2.4	0.7
3	15.0	5.0	2.2	1.1
4	17.8	2.8	2.1	0.7
5	8.1	3.2	1.6	0.9
Mean	11.9	3.5	2.1	0.9

The experimental conidtions were the same as in Fig. 3.

* The same as in Table I.

TABLE III

Effect of Glycine Concentration on the Incorporation

Glycine concentration (m*M*)	Glycine in μM per g. protein per 1.5 hours			
	2.04	0.68	0.185	0.09
Antibody	32.0	12.5	3.0	~
Sol-P*	7.2	5.5	2.1	0.3
RN-P*	6.2	3.1	0.7	0.3
DN-P*	2.7	1.7	0.4	0.1

The experimental conditions were the same as in Fig. 3, except that various concentrations of glycine were used.

* The same as in Table I.

DISCUSSION

In recent years it has become possible to approach the problem of the synthesis of tissue protein by the use of suitable isotopic amino acids and since then a great number of reports concerning this problem have been published. But only few investigations were made concerning the incorporation of labeled amino acids into the protein of lymphoid tissue. Using the cell suspension of the lymphosarcoma and spleen,

TABLE IV (1)

Effects of Anaerobiosis on the Incorporation by Cell Suspensions

Gas phase	O_2		N_2	
Addition	None	Glucose	None	Glucose, ATP
Antibody	8.75	8.45	0.063	0.087
Sol-P	2.55	1.85	0.011	0.027
RN-P	2.08	2.20	0.010	0.032
DN-P	0.62	1.07	0.016	0.018
Lactic acid content per flask after the incubation γ	238	880	600	1130

TABLE VI (2)

Gas phase	O_2	N_2		
Addition	None	None	ATP, creatine-phosphate	Glucose, ATP, creatine-phosphate, HDP
Antibody	13.5	0.038	0.057	—
Sol-P*	4.4	0.017	0.025	0.041
RN-P*	1.9	0.016	0.017	0.031
DN-P*	1.0	0.040	—	0.080
Lactic acid content per flask after the incubation γ	65	400	580	1580

The final concentration of glucose, ATP, creatine phosphate and hexose-diphosphate were 47 m*M*, 1 m*M*, 11.7 m*M* and 1.12 m*M*, respectively.

Other experimental conditions were the same as in Fig. 3.

The figures represents the incorporation expressed as μM per g. protein per 90 minutes.

* The same as in Table I.

Farber, Kit and Greenberg (*16, 18*) studied the incorporation of C^{14}-amino acid under various conditions, and Kit and Barron (*31*) demonstrated the effects of corticoid upon the incorporation of C^{14}-glycine into the protein of lymphoid tissues.

From the data represented in Fig. 3 and Table II, it can be seen

TABLE V

The Effect of Metabolic Inhibitors on the Rate of Incorporation by Cell Free Homogenate

Addition	Antibody	Sol-P	RN-P
None	100	100	100
Arsenate (10^{-3} *M*)	24	51	36
Monoiodoacetate (10^{-3} *M*)	51	18	39
None	100	100	100
2,4-Dinitrophenol (10^{-3} *M*)	58	44	
Malonate (1.2×10^{-3} *M*)	33	46	

Incorporation expressed as per cent of that without inhibitor. The experimental conditions were the same as those in Fig. 4.

that the rate of incorporation of C^{14}-glycine into protein fraction of immunized tissue, especially that into antibody is very high, as compared with the figures of the rate of the incorporation of C^{14}-amino acids into the lymphosarcoma and spleen (*16, 18*) or into other tissue proteins reported by other investigators using intact tissue slices or suspensions (*21–23, 28, 29*)[2]. This result may clearly demonstrate that the antibody is rapidly synthesized in the tissue of popliteal lymph nodes of the locally immunized animal, and agrees with the immunological investigations of Ogata and Mochizuki (*5*).

The fact that the rate of C^{14}-glycine uptake into antibody is much higher than that into other protein fractions, suggests that the other tissue proteins are not direct precursors of antibody, though the further fractionation of the soluble protein was not carried out in this experiment.

The data of the comparison of the rate of the incorporation into protein of different cellular fractions show clearly that the microsome protein incorporates amino acids at a much higher rate than any other cellular fractions. This result is in agreement with that of Hultin's (*19*) *in vivo* experiments and Siekevitz's (*20*) *in vitro* experiments using liver homogenates, though in the latter case the incorporation into the supernatant exhibits the lowest value. Very recently, after the finish-

2) For the incorporation rate in various tissue proteins readers are refered to Tarver (1954) (*27*)).

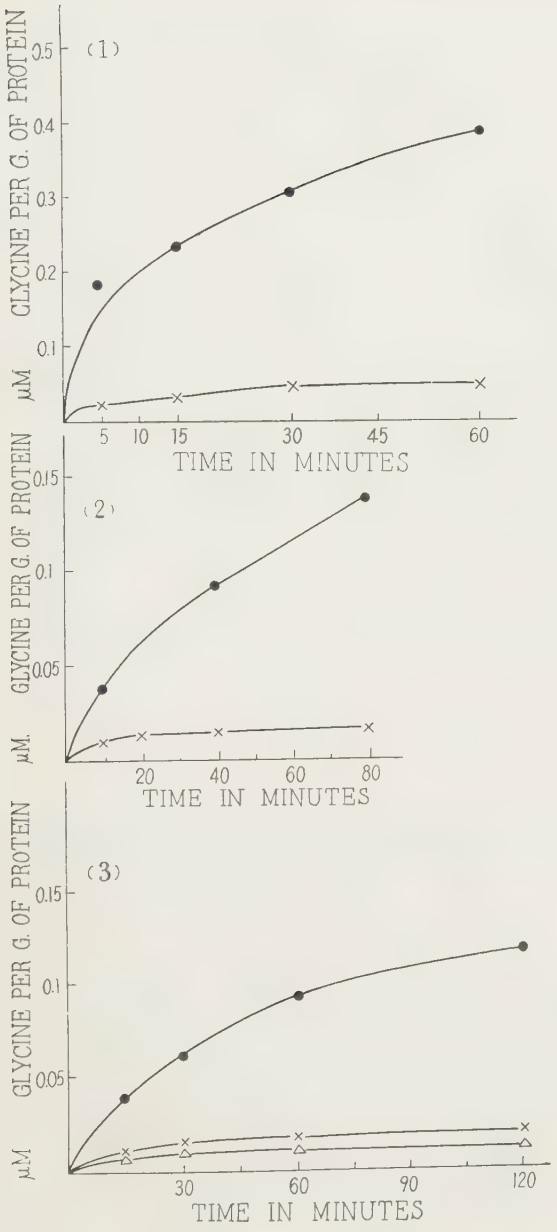


FIG. 4. Incorporation of glycine into antibody and other tissue proteins by the cell free homogenate *versus* time.

The homogenization medium contained $MgCl_2$ 0.004 *M*, K phosphate buffer (pH 7.4) 0.04 *M*, KCl 0.12 *M*. Nicotinamide (0.01 *M*) was added further in the case of Fig. 4 (1).

Each manometric flask contained 1.0 ml. of cell free homogenate, 0.2 ml. of 0.9 per cent NaCl, 0.1 ml. of C^{14}-glycine (specific activity, 38.5 μC./mg.). The concentration of C^{14}-glycine was 1.27 μM. Incubation at 37°, in oxygen.

ment of this work, Ziegler and Melchior (*25*) obtained similar results with ours, by cellular fractionation of liver slices after the incubation with S^{35}-methionine.

The incorporation of C^{14}-glycine into antibody and other protein fractions was shown to be greatly inhibited by anaerobiosis. Anaerobiosis has been reported to produce complete or variable inhibition of C^{14}-amino acid uptake into several tissue proteins (*18*, *21*). But recently Rabinovitz *et al.* (*24*) reported that the incorporation of C^{14}-amino acids into the protein of Ehrlich ascites carcinomatosa was supported anaerobically when sufficient glucose presented and active glycolysis occurred. In the present experiment the addition of glucose and ATP, and other P-compounds stimulated remarkably the production of lactic acid by cell suspensions, while restored the C^{14}-glycine incorporation only slightly as compared with the results of Rabinowitz *et al.* This discrepancy may be due to the difference between cell types studied. Further, using cell suspension or cell free homogenate system the detrimental effects of the inhibitors of respiration or phosphorylation were demonstrated. In addition it was indicated that the oxygen uptake stood in very intimate relation to the most suitable incorporation of glycine into antibody. Thus it may be concluded that in the tissue of the popliteal lymph nodes the aerobic energy yielding reaction may be absolutely necessary for the amino acid uptake into tissue protein and especially into antibody.

SUMMARY

Following the injection of crystalline ovalbumin into the foot-pads of rabbits, the popliteal lymph nodes were removed and the incorporation of 1-C^{14}-glycine into antibody and other cellular protein fractions was studied, using cell suspensions or cell free homogenates.

The following results were obtained.

1. Under suitable aerobic conditions the cell suspensions of the lymph nodes from immunized rabbits incorporated very rapidly C^{14}-glycine into antibody. The rates of incorporation of glycine into antibody, soluble protein, the protein moiety of ribonucleoprotein and that of desoxyribonucleoprotein, expressed as μM glycine per g. protein per hour, were 11.9, 3.5, 2.1, and 0.9, respectively, when glycine concentration was 0.68 m*M*.

2. The rate of the C^{14}-glycine uptake into antibody was higher than the proteins of the cellular components, fractionated by the pro-

cedure of Schneider, of which that of the microsome fraction showed the highest value, that of the soluble fraction the next, and protein moiety of desoxyribonucleoprotein the lowest value.

3. Aresenate, azide, dinitrophenol, monoiodoacetate and anaerobiosis inhibited the incorporation of glycine into antibody as well as into other cellular proteins. Under anaerobic conditions, the addition of glucose, ATP, and other P-compounds restored only slightly the C^{14}-glycine uptake.

4. When the cell free homogenate was used, the rate of the incorporation was greatly depressed. But even in this condition, the isotope uptake into antibody was more rapid than that into other protein fractions. The arsenate, monoiodoacetate, 2.4-dinitrophenol, or malonate inhibited the amino acid uptake in the cell free homogenate system.

This work was supported by a Grant in Aid for Scientific Research from the Department of Education.

The authors express sincerely their gratitude to Prof. Masuo Ogata and Issei Otawara for their advice and encouragement during this work.

REFERENCES

(*1*) Ehrlich, W. E., and Harris, T. N., *J. Exptl. Med.*, **76**, 335 (1942)
(*2*) Ehrlich, W. E., Harris, T. N., and Mertens, E., *J. Exptl. Med.*, **83**, 373 (1946)
(*3*) Harris, T. N., and Harris, S., *J. Exptl. Med.*, **90**, 169 (1949)
(*4*) Harris, T. N., and Harris, S., *J. Immunol.*, **61**, 193 (1949)
(*5*) Ogata, M., and Mochizuki, Y., *Seibutsu-Butsuri-Kagaku*, **2**, 226 (1955) (in Japanese)
(*6*) Heidelberger, M., and Kendall, F. E., *J. Exptl. Med.*, **50**, 809 (1929)
(*7*) Heidelberger, M., Kendall, F. E., and Soo Hoo, C. M., *J. Exptl. Med.*, **58**, 137 (1933)
(*8*) Heidelberger, M., and Kendall, F. E., *J. Exptl. Med.*, **62**, 697 (1935)
(*9*) Heidelberger, R., Schoenheimer, S., Ratner, S., and Rittenberg, D., *J. Biol. Chem.*, **144**, 541, 555 (1942)
(*10*) Roberts, S., Adams, E., and Whith, A., *J. Biol. Chem.*, **174**, 379 (1948)
(*11*) Ranney, H. M., and London, I. M., *Federation Proc.*, **10**, 562 (1951)
(*12*) Askonas, B. A., and Humphrey, J. H., *Biochem. J.*, **60**, x (1955)
(*13*) Potter, V. R., and Elvehjem, C. A., *J. Biol. Chem.*, **114**, 495 (1936)
(*14*) Folin, O., and Ciocalteau, V., *J. Biol. Chem.*, **73**, 627 (1929)
(*15*) Griffin, A. G., Nyle, W. N., Noda, L., and Luck, J. M., *J. Gen. Physiol.*, **31**, 1225 (1947)
(*16*) Farber, E., Kit, S., and Greenberg, D. M., *Cancer Research*, **11**, 485 (1951)
(*17*) Barker, J. B., and Summerson, W. H., *J. Biol. Chem.*, **138**, 535 (1941)

(*18*) Kits, S., and Greenberg, D. M., *Cancer Research*, **11**, 495, 500 (1951)
(*19*) Hultin, T., *Exptl. Cell Research*, **1**, 37 (1950)
(*20*) Siekevitz, P., *J. Biol. Chem.*, **195**, 549 (1952)
(*21*) Frantz, I. D., Loftfield, R. B., and Miller W. W., *Science*, **106**, 544 (1947)
(*22*) Borsook, H., Deasy, C. L., Haagen-Smit, A. J., Keighley, G., and Lowy, P. H., *J. Biol. Chem.*, **186**, 297 (1950)
(*23*) Borsook, H., Deasy, C. L., Haagen-Smit, A. J., Keighley, G., and Lowy, P. H. *J. Biol. Chem.*, **196**, 669 (1952)
(*24*) Rabinovitz, M., Olson, M. E., and Greenberg, D. M., *J. Biol. Chem.*, **213**, 1 (1955)
(*25*) Ziegler, D. M., and Melchior, J. B., *J. Biol. Chem.*, **217**, 569 (1955)
(*26*) Schneider, W. C., *J. Biol. Chem.*, **176**, 259 (1948)
(*27*) Tarver, H., The Proteins, Academic Press Inc., Publishers, New York, p. 1229 (1954)
(*28*) Melchior, J. B., Dissertation, University of California, Berkley, (1946)
(*29*) Melchior, J. B., and Tarver, H., *Arch. Biochem.*, **12**, 309 (1947)
(*30*) Kekwick, R.A., and Cannan, R.K., Biochem. J., **30**, 227 (1936)
(*31*) Kit, S., and Barron, E.S.G., Endocrinology, **52**, 1 (1953)

The Journal of Biochemistry, Vol. 43, No. 5, 1956

BIOCHEMICAL STUDIES ON "COENZYME SULFATE ANALOGUES"

II. EFFECTS OF RIBOFLAVIN-MONOSULFATE ON BACTERIAL GROWTH

By FUJIO EGAMI, MARIKO NAOI, MITSUHIKO TADA*, and KUNIO YAGI*

(From the Department of Chemistry, Faculty of Science, Nagoya University and the Department of Biochemistry, School of Medicine, Nagoya University, Nagoya)*

(Received for publication, May 21, 1956)

Riboflavin-monosulfate (FMS) (*1*) has been shown to compete with flavin-adenine-dinucleotide (FAD), thus inhibiting the oxidative deamination of D-alanine by D-amino-acid oxidase (*2*). This finding led us to consider that FMS, as an antivitamin-B_2, might disturb the metabolic pattern and inhibit the growth of certain bacteria and higher animals. In this connection, the present paper deals with the effects of FMS on the growth of bacteria.

EXPERIMENTAL

Organisms Chosen—L. casei (ATCC. No. 7469) which requires riboflavin for growth, and *Str. faecalis* (ATCC. No. 8043) which does not require it.

Procedure—It was essentially the same as that for routine riboflavin assay. The medium of Flynn *et al.* (*3*) with the omission of riboflavin was used as a basal medium.

Inocula were prepared by the incubation of the stock culture in the basal medium at 37° for 24 hours. Then sufficient cells were suspended in sterilized distilled water to give a slight turbidity. One drop of the suspension was used as the inoculum.

Each 2 ml. of the basal medium were placed in the assay tubes, capped and autoclaved. One of a series of flavins was added after sterilization by Seitz filter. Each volume was adjusted to 4 ml. with distilled water. The tubes, after inoculation, were incubated at 37° for 48 hours. Bacterial growth was measured turbidimetrically with a spectrophotometer at 660 mμ. After 72 hours of incubation, organic acid produced was titrated with 0.05 N NaOH.

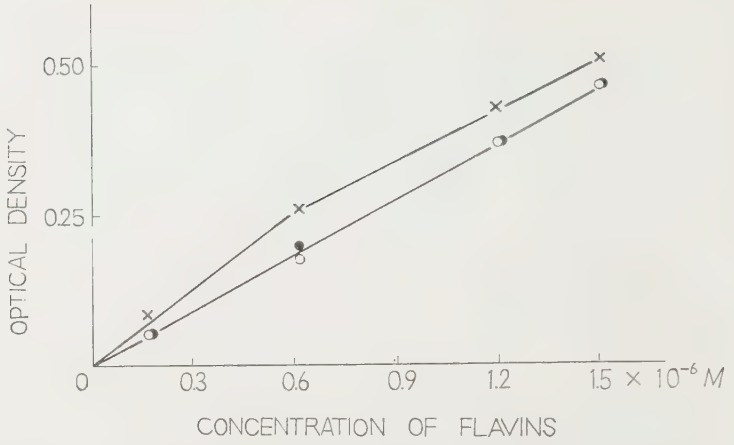


Fig. 1 a. Growth curve of *L. casei* in the presence of flavins.
Growth was measured by turbidimetry after 48 hours of incubation. (Expt. 1)
○—○ Riboflavin, ●—● FMN, ×—× FAD.

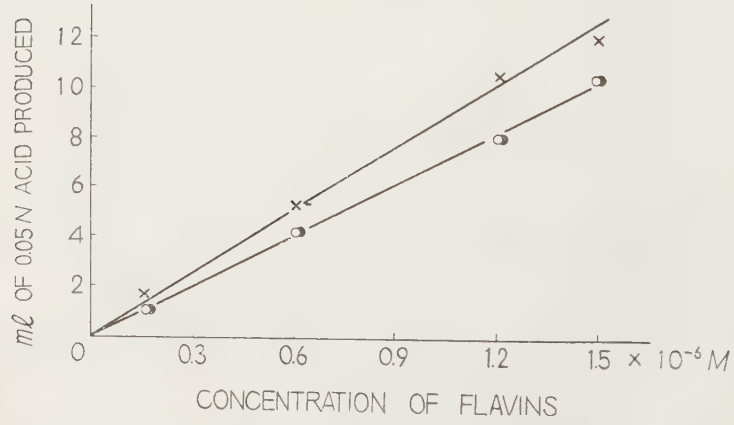


Fig. 1 b. Organic acid produced by *L. casei* in the presence of flavins. (Expt. 1)
Growth was measured by acidmetry after 72 hours of incubation.
○—○ Riboflavin, ●—● FMN, ×—× FAD

Growth Test with Flavins—One of the flavins (Riboflavin, FMN, FAD, or FMS) was added to the basal medium and the growth was

measured by turbidimetry and acidimetry. Experiments with *L. casei* (Expt. 1.) are represented in Fig. 1 a and 1 b.

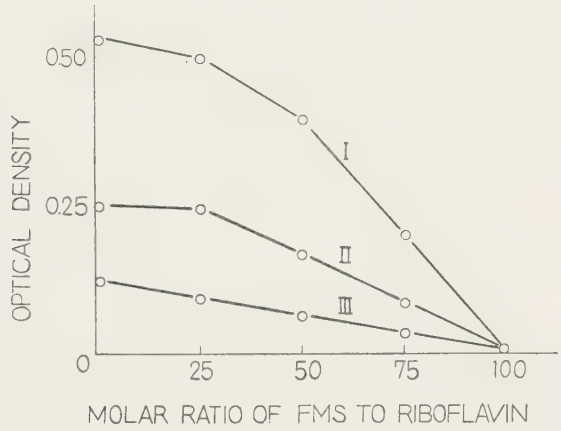


FIG. 2. Effect of FMS upon bacterial growth in the medium with riboflavin. (Expt. 2)

Riboflavin concentration: I, 1.5×10^{-6} *M*; II, 0.6×10^{-6} *M*; III, 0.3×10^{-6} *M*.

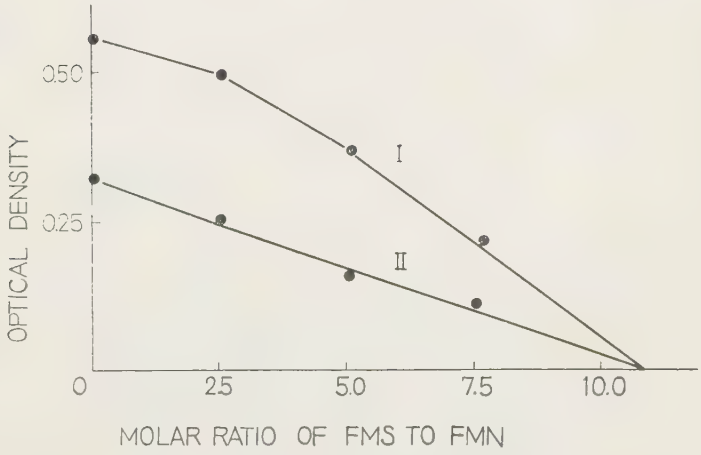


FIG. 3. Effect of FMS upon bacterial growth in the medium with FMN. (Expt. 3)

FMN concentration: I, 1.5×10^{-6} *M*; II, 1.0×10^{-6} *M*.

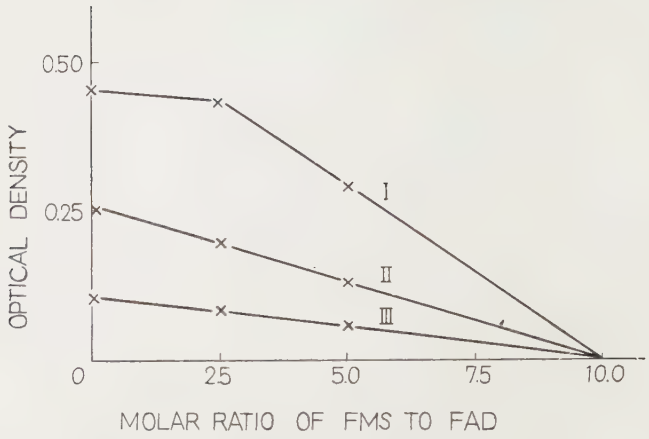


FIG. 4. Effect of FMS upon bacterial growth in the medium with FAD. (Expt. 3)

FAD concentration: I, 1.2×10^{-6} *M*; II, 0.6×10^{-6} *M*; III, 0.3×10^{-6} *M*.

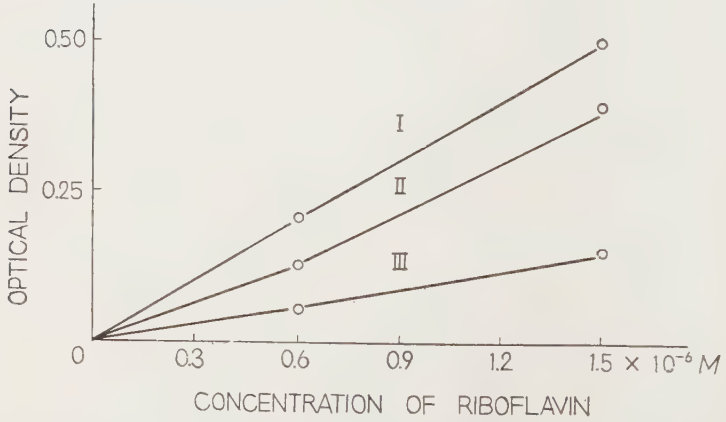


FIG. 5. Relation between riboflavin concentration and bacterial growth when molar ratio of FMS to riboflavin is constant. (Expt. 4)

Molar ratio of FMS to riboflavin: I, 0/1; II. 25/1: III, 75/1.

The bacterial growth was linear with concentration over the range from 0 to 1.5×10^{-6} *M* (0.05 μg./ml. as riboflavin) of FAD, FMN, and

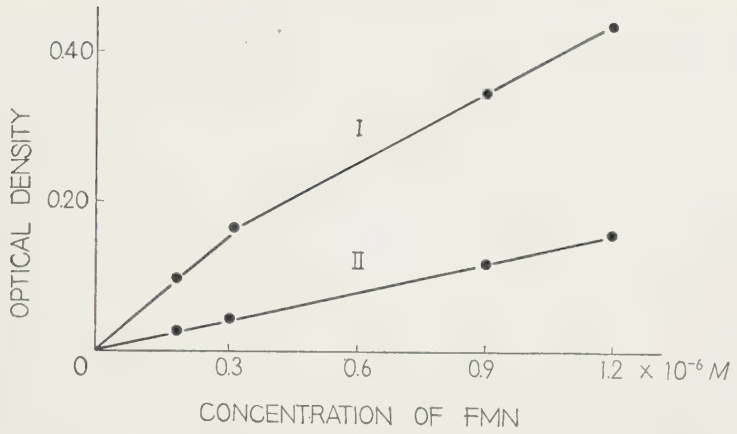


FIG. 6. Relation between FMN concentration and bacterial growth when molar ratio of FMS to FMN in constant. (Expt. 4)
Molar ratio of FMS to FMN: I, 0/1; II, 7.5/1.

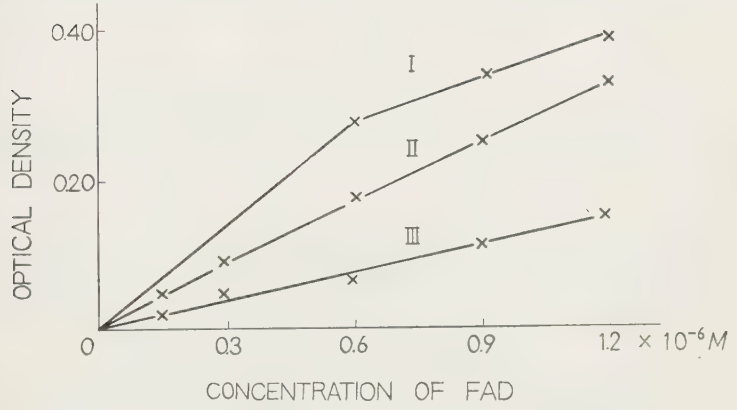


FIG. 7. Relation between FAD and bacterial growth when molar ratio of FMS to FAD is constant. (Expt. 4)
Molar ratio of FMS to FAD: I, 0/1; II, 2.5/1; III, 7.5/1.

riboflavin. FMS did not support growth at the same concentration level, or even in higher dose (10^{-3} *M*). With *Str. faecalis* (Expt. 2.), neither riboflavin, FMN, FAD, nor FMS affected growth at the concentration level up to 25 μg./ml. flavin.

Growth Inhibition by FMS—The effects of varying concentrations of FMS on the growth of *L. casei* in the presence of growth promoting concentration of riboflavin, FMN, or FAD were investigated (Expt. 3.). The antagonistic acitivity of FMS to the tested flavins in the bacterial growth is expressed by means of an "inhibition index." Such indices represent the molar ratio of FMS to growth promoting flavin at the point of half growth inhibition, the indices derived from the data summarized in Figs. 2, 3, and 4, being 5, 6 and 63 for FAD, FMN, and riboflavin, respectively. The high inhibition index for FMS-riboflavin system may be explained by the higher permeability of riboflavin.

Since a) FMS is a structural analogue of the growth promoting flavins, and b) there is a linear relation between growth and flavin concentration when FMS/flavin is constant and flavin concentration is low (Expt. 4., Figs. 5, 6, and 7), it may be reasonable to conclude that FMS is a competitive inhibitor for growth promoting flavins.

This growth inhibition is a bacteriostatic action, because *L. casei* was living even after 10 hours in the medium with FMS (0.5×10^{-3} *M*).

SUMMARY

1. Riboflavin-monosulfate (FMS) does not support the growth of *L. casei*.

2. It competes with riboflavin, FMN, and FAD, and when added to growth promoting concentration of these flavins, it inhibits the growth of *L. casei*.

3. Inhibition indices of FMS are 5, 6, and 63 for FAD, FMN, and riboflavin, respectively. The higher inhibition index found for riboflavin may be explained by the higher permeability of riboflavin.

4. The growth inhibition by FMS is a bacteriostatic action.

5. FMS has no effect on the growth of *Str. facalis*.

We acknowledge the gift of bacterial strains by the Institute for Infectious Diseases and the Institute of Applied Microbiology of the University of Tokyo.

REFERENCES

(*1*) The synthesis of FMS will be published in *J. Chem. Soc. Japan, Pure Chem. Sect.*, by N. Takahashi, K. Yagi, and F. Egami.

(*2*) Egami, F., and Yagi, K., *J. Biochem.*, **43**, 153 (1956)

(*3*) Flynn, L. M., Williams, V. B., O'dell, B. L., and Hogan, A. G., *Anal. Chem.*, **23**, 180 (1951)

The Journal of Biochemistry, Vol. 43, No. 5, 1956

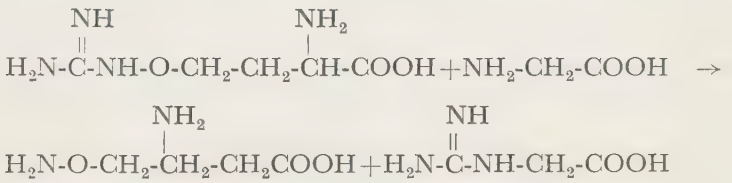
ENZYMATIC TRANSAMIDINATION FROM CANAVANINE TO GLYCINE BY HOG KIDNEY EXTRACTS

By SEIICHIRO NAKATSU

(*From the Cehmical Department, Faculty of Science, Kyushu University, Fukuoka*)

(Received for publication, May 31, 1956)

Borsook and Dubnoff (*1*) demonstrated that the amidine moiety of arginine was transferred to glycine to form glycocyamine by slices or cell-free macerates of mammalian kidneys, and designated the enzyme concerned "transamidinase." Walker (*2*) observed recently that the amidine moiety of canavanine was transferred to ornithine to form arginine by hog kidney extracts*. The present author has found that hog kidney extracts catalyze a transamidination reaction forming glycocyamine from canavanine and glycine as given in the accompanying diagram.



In this paper the general properties of this reaction are presented.

MATERIALS AND METHODS

Materials—L-Canavanine was isolated from jack bean (*3*), and other reagents used were commercial products. The buffers employed were Walpole's 1/5 *M* acetate (pH 4.0–5.0), Sørensen's 1/15 *M* phosphate (pH 5.9–8.0), and Atkins-Pantin's 1/10 *M* borate (pH 8.4–10.0).

Enzyme Solution—Hog kidney, purchased from a meat-shop, was ground in a mortar with sea sand and water (0.5 ml. per g. of the moist tissue), and was kept in an ice-box overnight under toluene. The mixture was centrifuged, and the supernatant liquid (somewhat turbid) was used as an enzyme solution.

* During the present author was making this paper after the completion of the work presented here, the Walker's report (*2*) appeared.

Incubation Procedure—Mixtures of canavanine, glycine, buffer, and the enzyme were incubated under toluene at 35°. After incubation, 0.25 volumes of 2 *M* acetic acid containing 10 per cent sodium chloride was added to each of the reaction mixtures, and then the mixtures were heated for 5 minutes in a boiling water-bath. To remove the coagulated protein, the mixtures were filtered after cooling, and the filtrates were used for the determination of glycocyamine.

Determination of Glycocyamine—Glycocyamine is the only substance in the reaction mixtures which gives a positive test with the Sakaguchi reagent, but, since the large amounts of canavanine and glycine interfere with the determination of glycocyamine by this reagent, it is necessary to separate glycocyamine from these two amino acibs. The separation was achieved by paper chromatography.

The chromatograms were developed by the descending technique on filter paper (Toyo-Roshi No. 52, 2×40 cm.) with a butanol-acetic acid-water mixture (4:1:1), and were sprayed with the Sakaguchi reagent to detect guanidino groups, or with ninhydrin to detect primary amino groups. Rf values of glycocyamine, glycine, and canavanine were 0.30, 0.18, and 0.13, respectively. Quantitative chromatograms were run usually with a batch of eleven sheets of paper in a tank, one sheet of which was sprayed with the Sakaguchi reagent for locating glycocyamine. From the other paper strips the areas corresponding to the spot of glycocyamine were cut off, and the glycocyamine so separated was extracted by heating the paper with 10 ml. of water in a stoppered tube at 70–80° for 30 minutes. Taking 5 ml. of the supernatant liquid of the extract, glycocyamine was determined by the Sakaguchi's colorimetric method (*4*).

RESULTS

Formation of Glycocyamine

When canavanine and glycine were incubated with the extract of hog kidney, a substance was formed which gave a red color with the Sakaguchi reagent. This substance had always the same Rf values as an authentic glycocyamine when developed on filter paper with various solvents. Consequently, it seems sure that the substance formed is glycocyamine.

The effect on the reaction of omitting one of the components is shown in Table I. If the enzyme solution was omitted from the reaction mixture, no glycocyamine was formed. A small amount of glycocyamine was found to be present in the extract of kidney. A very slight increase in glycocyamine was observed when glycine alone was incubated with the enzyme. Canavanine without glycine led to a somewhat significant increase in glycocyamine. When both amino acids were added together, the amount of glycocyamine formed was more than

4 times the amount with canavanine alone. From these results it is sure that the enzymatic canavanine-glycine transamidination reaction as described in the introduction occurs. The increase obtained with canavanine alone may indicate the presence of a small amount of free glycine in the kidney extract. Both α- and β- alanines were inactive as acceptors for amidine groups.

Table II shows the relation between time of incubation and the amount of glycocyamine formed.

TABLE I

Formation of Glycocyamine

Canavanine (μM)	Glycine (μM)	Enzyme soln. (*ml.*)	Glycocyamine found (μM)
40	80	1	10.3
40	0	1	3.1
0	80	1	1.5
0	0	1	1.0
40	80	0	0

Total volume 4 ml., buffer (pH 7.4) 3 ml., temp., 35°, time of incubation 4 hrs.

TABLE II

Rate of Glycocyamine Formation

Time of incubation	*hrs.*	2	4	24
Glycocyamine formed	μM.	2.5	9.9	25.4

Total volume 4 ml., buffer (pH 7.4) 3 ml., enzyme soln. 1 ml., canavanine 40 μM., glycine 80 μM., temp. 35°.

Effect of pH on Activity of Transamidinase

The effect of pH on the glycocyamine formation is shown in Table III. The reaction proceeded at a significant rate between pH 5.9 and 9.0, with an optimum in the neighborhood of pH 7.4 under the conditions employed.

TABLE III

Effect of pH

pH	5.0	5.9	7.0	7.4	8.0	8.5	9.0	10.0
Glycocyamine formed μM.	0	3.9	9.1	11.6	9.8	6.7	4.5	0

Total volume 4 ml., buffer 2 ml., enzyme soln. 1 ml., canavanine 40 μM, glycine 80 μM, temp. 35°, time of incubation 4 hrs.

Effect of Canavanine Concentration on Glycocyamine Formation

The effect of the canavanine concentration on the glycocyamine formation is shown in Table IV. The rate of glycocyamine formation increased with increasing canavanine concentration in the range of 0.0005–0.005 *M*, but remained almost constant at the concentrations higher than 0.005 *M*. This observation may be explained by the Michaelis' theory.

TABLE IV

Effect of Canavanine Concentration

Canavanine concentration *M*	0.0005	0.001	0.002	0.005	0.01	0.02	0.05
Glycocyamine formed μM.	2.0	4.3	6.5	11.2	9.7	10.2	11.4

Total volume 4 ml., buffer (pH 7.4) 3 ml., enzyme soln. 1 ml., glycine 0.02 *M*, temp. 35°, time of incubation 4 hrs.

Effect of Glycine Concentration on Glycocyamine Formation

As shown in Table V, the rate of glycocyamine formation increased approximately in proportion to the concentration of glycine in the range of 0.001–0.01 *M*. However, the rate remained almost constant at the concentrations of glycine higher than 0.02 *M*.

Effect of Dialysis on Enzyme Solution

The activities of the following enzyme solutions are compared each other in Table VI.

TABLE V

Effect of Glycine Concentration

Glycine cencentration *M*	0.001	0.002	0.005	0.01	0.02	0.05	0.1
Glycocyamine formed μM	1.0	2.1	4.6	9.9	13.4	12.1	13.0

Total volume 4 ml., buffer (pH 7.4) 3 ml., enzyme soln. 1 ml., canavanine 0.01 *M*, temp. 35°, time of incubation 4 hrs.

(i) An enzyme solution was dialyzed for 2 days in the presence of toluene against running water (Enzyme solution 1).

(ii) To a dialyzed enzyme solution, sufficient manganese sulfate was added to give a concentration of 2 mg. Mn^{++}/ml. (Enzyme solution 2).

(iii) An enzyme solution which had not been subjected to dialysis was diluted with the amount of water equivalent to the expansion of volume of Enzyme solution 1 during dialysis (Enzyme solution 3).

TABLE VI

Effect of Dialysis

Enzyme solution	1	2	3
Glycocyamine formed μM	6.4	3.7	3.9

Total volume 4 ml., buffer (pH 7.4) 3 ml., enzyme soln. 1 ml., canavanine 40 μM, glycine 80 μM, temp. 35°, time of incubation 4 hrs.

As seen in Table VI, the activity of transamidinase was increased by dialysis, and was decreased by the addition of manganese sulfate, in contrast with canavanase whose activity was enhanced by manganese ions. The increase of the activity of transamidinase by dialysis may be attributed to the removal of inhibitors, probably heavy metal ions, because the activity of the enzyme was increased also by the addition of a metal binder, ethylenediaminetetraacetate.

Inhibition by p-Chloromercuribenzoate

The activity of transamidinase was reduced to about 60 per cent

by the addition of *p*-chloromercuribenzoate to give a final concentration of 0.001 *M*, but was restored completely by the treatment with an excess amount of glutathione (final concentration 0.01 *M*). These results are shown in Table VII. Because *p*-chloromercuribenzoate is known to react specifically with sulfhydryl groups, it is suggested that sulfhydryl groups may be involved in the enzyme action of transamidinase. This suggestion is also supported by the sensitiveness of the enzyme to heavy metal ions described above.

TABLE VII

Inhibition by p-Chloromercuribenzoate

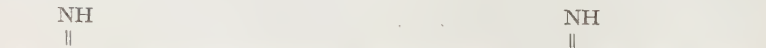
Enzyme soln.	Without *p*-chloromercuribenzoate	Inhibited by *p*-chloromercuribenzoate	Reactivated by glutathione
Glycocyamine formed μM	10.4	6.1	11.0

Total volume 4 ml., buffer (pH 7.4) 3 ml., enzyme soln. 1 ml., canavanine 40 μM, glycine 80 μM, temp. 35°, time of incubation 4 hrs.

DISCUSSION

It seems unlikely at present that canavanine is a normal metabolite of higher animals. It is probable, however, that plants containing canavanine are ingested by higher animals. Consequently, it is possible that the canavanine-glycine transamidination reaction occurs in higher animals and canavanine replaces arginine so far as the glycocyamine formation is concerned.

The mechanism of transamidination reaction has not been made clear. However, it was suggested by the results presented here that that sulfhydryl groups might be involved in the enzyme action and no cofactor might be required. On the other hand, glycocyamine can be synthesized by the action of *S*-methylisothiourea on glycine in a weakly alkaline aqueous solution at a room temperature as given in the following diagram:



Therefore, it is reasonable to speculate that an enzyme-amidine intermediate complex of isothiourea-type may be formed, and the mechanism of the reaction may be tentatively represented by the following diagram:

$$\text{Canavanine} + \text{H-S-enzyme} \rightarrow \text{canaline} + \text{H}_2\text{N-}\overset{\overset{\text{NH}}{\|}}{\text{C}}\text{-S-enzyme}$$

$$\text{H}_2\text{N-}\overset{\overset{\text{NH}}{\|}}{\text{C}}\text{-S-Enzyme} + \text{glycine} \rightarrow \text{H-S-enzyme} + \text{glycocyamine}$$

Since arginine-handling enzymes of many organisms cannot distinguish completely between arginine and canavanine, the enzyme catalyzing the transamidination from canavanine may be the same as that catalyzing the transamidination from arginine. Borsook and Dubnoff reported that a cell-free macerate of kidney was less active than an equivalent amount of kidney tissue in the form of slices (*1*). This observation may be explained by the sensitiveness of the enzyme to heavy metal ions reported in this paper; the destruction of the cell may involve contamination of the enzyme with various metal ions from which the enzyme is presumably separated in the intact cell.

SUMMARY

An enzyme from hog kidney was found to catalyze the transfer of the amidine moiety of canavanine to glycine with the formation of glycocyamine. The enzyme was sensitive to heavy metal ions, and was inhibited by *p*-chloromercuribenzoate. No cofactor requirement was observed, and the optimal pH was about 7.4. It was suggested that an isothiourea-type enzyme-amidine intermediate complex might be formed.

The author wishes to thank Dr. S. Shibuya and Dr. N. Izumiya for their valuable advice.

REFERENCES

(*1*) Borsook, H., and Dubnoff, J. W., *J. Biol. Chem.*, **138**, 389 (1941)
(*2*) Walker, J. B., *J. Biol. Chem.*, **218**, 549 (1956)
(*3*) Damodaran, M., and Narayanan, K. G. A., *Biochem. J.*, **33**, 1740 (1939)
(*4*) Sakaguchi, S., *J. Biochem.*, **37**, 231 (1950)

The Journal of Biochemistry, Vol. 43, No. 5, 1956

DIARYL PYROPHOSPHATASE AND FAD PYROPHOSPHATASE

By SHIGERU YAMAWAKI

(*From the Department of Biochemistry, School of Medicine, Chiba University, Chiba*)

(Received for publication, June 6, 1956)

It was reported (*1*) that diphenyl pyrophosphate could be hydrolysed by inorganic pyrophosphatase into 2 moles of monophenyl phosphate when a cofactor present in a heated kidney autolysate is added. The present experiment has shown that diphenyl pyrophosphate can be split at the pyrophosphate linkage by a liver enzyme preparation not containing inorganic pyrophosphatase. The action of this enzyme, diaryl pyrophosphatase, when purified, is specific to diphenyl pyrophosphate. It is inactive to flavin adenine dinucleotide, which, however, is hydrolysed by muscle enzyme to produce flavin monophosphate and adenylic acid. The latter enzyme seems to belong to the nucleotide pyrophosphatase of Kornberg (*2*) and is inactive to diphenyl pyrophosphate. The preparation and properties of these enzyme solutions are described here.

EXPERIMENTAL AND RESULTS

Measurement of Hydrolysis at Pyrophosphate Linkage—To estimate the extent of diphenyl pyrophosphate hydrolysis at the pyrophosphate linkage, the monophenyl phosphate produced should be quantitatively split by monoesterase into phenol and inorganic phosphate which are colorimetrically measurable by the indophenol method (*3*) and Fiske-Subbarow's or Youngburg's method, respectively. As an effective monoesterase a human urine sample simply dialysed was used. Activity of urine monoesterase at its optimum pH 5 was strong enough to completely hydrolyse monophenyl phosphate of 0.001 *M* concentration in two hours, whereas in the same conditions inorganic and diphenyl pyrophosphates used as substrates remained intact. Potassium diphenyl pyrophosphate was prepared according to the procedure of Neuberg and Wagner (*4*).

Preparation of FAD Solution—It was prepared from guinea pig liver according to the warm water extraction method of Yagi (*5*) and the phenol-ether method of Warburg and Christian (*6*). A slight modification was made in following points. The liver extract for phenol extraction of flavin compounds was obtained by heating liver slices in a mixture (1 : 5) of acetate buffer (pH 5) and physiological saline solution for 5 minutes at 80°, homogenising the coagulated tissue debris, heating again for 5 minutes, and centrifuging and the supernatant fluid was, prior to phenol treatment, saturated with sodium chloride instead of ammonium sulfate, since the contamination of the latter caused an inhibition of enzymatic FAD hydrolysis. Total flavin content of the solution thus prepared was estimated by the method of Fujita-Yagi (*5*) after conversion to lumiflavin. Flavin compounds remained in the water layer after three times extraction with benzyl alcohol were assumed to be flavin mononucleotide (FMN) and FAD. For estimation of FAD, the solution was incubated at pH 5 with urine monoesterase for five hours to hydrolyse FMN to flavin riboside (FR), then extracted three times with benzyl alcohol to make free from FR, and the FAD remained was fluorometrically estimated by the method of Fujita-Yagi (*5*) after conversion to lumiflavin. The complete hydrolysability of FMN under the conditions mentioned above was ascertained from the result that FMN (Takeda) solution incubated with monoesterase and treated with benzyl alcohol did not produce any lumiflavin. FAD suffered no decrease by urine enzyme. FAD content of the solution thus prepared and used for the experiments was 3 to 5 μg. per ml. and a trace of FMN and FR was present.

The Test of FAD Pyrophosphatase Activity—It was carried out as in the case of diphenyl pyrophosphatase in two steps: Two ml. of FAD solution, 2 ml. of FAD pyrophosphatase, and 2 ml. of buffer solution were mixed in a brown glass tube, covered with toluene, and incubated for the time as indicated in the experimental results. For removal of FMN at the second step of the procedure, the mixture was adjusted to pH 5 by adding N NaOH or acetic acid, 1 ml. of acetate buffer of pH 5 was added, and the volume was made to 9 ml. with water. It was heated at 80° for 15 minutes to stop the enzyme action and then incubated with 2 ml. urine monoesterase solution at 37° for 2 hours. FR produced was removed by benzyl alcohol extraction and FAD remaining unhydrolysed was estimated fluorometrically after conversion to lumiflavin.

Preparation of Liver Diaryl Pyrophosphatase—Pig liver brei was autolysed with 2 volumes of water at 37° for 3 days and the filtrate was dialysed for 2 days against running water. This solution which contained monoesterase and inorganic and diaryl pyrophosphatases was acidified to pH 5 by addition of dilute acetic acid, the protein precipitated was centrifuged off, and the supernatant solution was neutralized to pH 9 by addition of 0.1 *N* NaOH, heated at 65° for ten minutes, and dialysed. The solution thus treated did not hydrolyse monophenyl phosphate and inorganic pyrophosphate in pH range from 3.2 to 9.0. It caused by its single action in the same pH range no liberation of phenol or inorganic phosphate from diphenyl pyrophosphate. However, when the diphenyl pyrophosphate solution incubated for some time with the enzyme solution was heated at pH 5 and 100° for five minutes to inactivate any enzyme present in the test mixture and then reincubated with urine monoesterase, the production of equivalent amounts of free phenol and inorganic phosphate was observed.

Optimum pH of Diaryl Pyrophosphatase—It was measured by the two-step procedure. The presence of two isodynamic, acid and alkaline, types was thereby revealed (Table I). Calcium or magnesium ion showed neither activation nor inhibition of the liver diaryl pyrophosphatase.

TABLE I

Hydrolysis of Diphenyl Pyrophosphate

pH	3	4	5	6	7	8	9
Hydrolysis per cent	9	18	13	12	16	16	12

Mixture of 1 ml. *M*/200 diphenyl pyrophosphate, 2 ml. of diluted diaryl pyrophosphatase, and 2 ml. buffer (*M*/10 acetate or Veronal) was incubated for two hours. After adjusting to pH 5, 1 ml. of acetate buffer of pH 5 was added and the total volume was made to 8 ml. by the addition of water. The mixture was heated for 5 minutes at 100°, cooled to 37°, and incubated with 2 ml. urine monoesterase for two hours. Phenol liberation was measured. Production of 2 moles phenol corresponds to 100 per cent hydrolysis.

Liver diaryl pyrophosphatase solution so long as treated as described above, still contained an alkaline FAD pyrophosphatase, since two ml. of it completely hydrolysed 11 μg. FAD at pH 8 to 9 to FMN

in 2.5 hours, while at pH 5 the hydrolysis proceeded to the extent of 50 per cent, and at pH 3 and 4, almost none. The diaryl pyrophosphatase solution could be made free from this alkaline FAD pyrophatase as follows.

Purification of Diaryl Pyrophosphatase—The enzyme solution heated at pH 9 as metioned above was acidified with 0.1 *N* hydrochloric acid to pH 3 and heated for 10 minutes in a water bath of 75°. After adjusting to pH 5.6 by the addition of 0.1 *N* NaOH and leaving in an ice-box over night, the precipitate produced was suspended in 0.05 *M* Veronal buffer of pH 9, kept at 37° for 3 hours, and centrifuged. The clear supernatant solution obtained was proved to retain the diaryl pyrophosphatase action and presented 52 per cent and 46 per cent hydrolysis of diphenyl pyrophosphate in two hours at pH 5 and 8, respectively, but showed no FAD hydrolysis at all.

From the results mentioned above it should be concluded that diaryl pyrophosphatase and FAD pyrophosphatase are different enzymes. Then preparation of FAD pyrophosphatase free from diaryl pyrophosphatase activity was desirable.

Muscle FAD Pyrophosphatase—Rabbit muscle was autolysed with 2 volumes of water at 37° for 4 days and the filtrate was dialysed. This solution was unable to produce any phenol from diphenyl pyrophosphate at pH 5 and 9, even after successive action at pH 5 of urine monoesterase, and therefore, it was free from diaryl pyrophosphatase. However, it exhibited, in case of no further purification, some activity of monoesterase and inorganic pyrophosphatase at pH 5 and 9. Since it has been proved that alkaline FAD pyrophosphatase of the liver was resistant to heating for 10 minuts at 65° and pH 9, the muscle autolysate was treated in a similar way. The solution thus heated, neutralised, and dialysed did not contain any monoesterase and inorganic pyrophosphatase. Optimum pH of FAD hydrolysis was 8 as illustrated in Table II. Alka-

TABLE II

FAD Pyrophosphatase of Rabbit Muscle

pH	3	4	5	6	7	8	9
Hydrolysis per cent	3	5	16	17	100	100	78

Two ml. of FAD solution (4.6 μg. per ml.) was used. Reaction time for the first step at various pH was 18 hours. Reaction time for the second step with urine monoesterase at pH 5 was 5 hours.

line FAD pyrophosphatase activity was not influenced by Ca or Mg ions.

Paper Chromatography of Hydrolytic Products of FAD—FAD solution was incubated with muscle FAD enzyme at pH 8 for 24 hours. After adjusting to pH 5, it was saturated with ammonium sulfate, extracted with phenol, and riboflavin compounds in the phenol layer were transferred as usual to 0.1 ml. of water for chromatography. Toyo filter paper No. 50 and the upper layer of a mixture of *n*-butanol, acetic acid, and water (4 : 1 : 5) was used. No fluorescent spot of FAD (Rf 0.02) was visible under a mercury lamp and instead, a very intense spot of FMN (Rf 0.09) appeared. However, when the FAD hydrolysate was successively incubated with urine monoesterase and then concentrated by the phenol procedure, a single fluorescent spot was observed at Rf 0.30, indicating the presence of riboflavin produced by hydrolysis of FMN, while no spots of other Rf were visible. The production of FMN from FAD was thus verified. If FAD were to be split at the pyrophosphate linkage, adenylic acid should be the other product, which could be detected by the following procedure. Ten aliquot samples of FAD hydrolysate of pH 8 by muscle FAD pyrophosphatase were acidified to pH 5 and heated at 80° for 15 minutes. The combined filtrate was concentrated to 1 ml. at 80° for application to paper chromatography. Under an ultraviolet irradiation with the Matsuzaki's apparatus (*7*), a dark apot of adenosine 5′-phosphate was visible, Rf of which was 0.65 and 0.71, when developed with a mixture of 5 per cent potassium dihydrogen phosphate and isoamyl alcohol and of 5 per cent disodium hydrogen phosphate and isoamyl alcohol, respectively.

Action of Potato Phosphatase on Diphenyl Pyrophosphate and FAD—Kornberg (*2*) isolated nucleotide pyrophosphatase from potatos. In the present experiment, the purification of potato enzymes according to his method remained at the first fractionation with ammonium sulfate and the precipitate obtained was dissolved in water and dialysed. This enzyme solution hydrolysed diphenyl pyrophosphate at the optimum pH of 3. Besides this acid enzyme there were, however, an acid monoesterase and two, acid and alkaline, inorganic pyrophosphatase. The solution was, therefore, heated as usual at 65° and pH 9 for 10 minuts and dialysed. Hydrolysis of FAD by this dialysed solution proceeded optimally at pH 3, the higher the pH of the reaction mixture the more FAD remained unchanged.

DISCUSSION

Diphenyl pyrophosphate was hydrolysed into two moles of monophenyl phosphate by an enzyme, provisionally named diaryl pyrophosphatase, without any liberation of free phenol. Successive action of urine monoesterase produced phenol and inorganic phosphate in equivalent amounts. Phosphodiesterase, monoesterase, and inorganic pyrophosphatase had no action on that substrate. Kurata (*1*) reported twenty years ago that the inorganic pyrophosphatase could hydrolyse diphenyl pyrophosphate when an activator was present. The diaryl pyrophosphatase in the present experiment was free from inorganic pyrophosphatase and seemed to need no activator. Whether there are two different enzymes for the hydrolysis of pyrophosphate linkage of diphenyl pyrophosphate remains at present undetermined. The nomenclature of diaryl pyrophosphatase was preferred, since it was inactive to FAD. There are acid and alkaline diaryl pyrophosphatases in the liver, the optimum pH being 4 and 8, respectively. The acid enzyme free from the alkaline isodynamic enzyme can be prepared from a potato extract.

FAD was, on the other hand, hydrolysable by a specific FAD pyrophosphatase. An alkaline FAD pyrophosphatase of the muscle could be prepared free from other phosphatases, whereas an acid FAD pyrophosphatase was found in a potato extract. It can be said that there are also two isodynamic types of FAD pyrophosphatase.

Since diaryl and FAD pyrophosphatases are different from each other, specificity of the enzymes capable of splitting pyrophosphate linkage depends upon the sort of hydroxyl compounds esterified by phosphoric acid. The results reported by Kornberg (*2*) that DPN, TPN, and FAD are hydrolysed by a single nucleotide pyrophosphatase is reasonable. According to this author the optimum pH of DPN hydrolysis by potato enzyme is 8 and that of FAD hydrolysis seems to be near this pH range. In the present esxperiment, however, an acid FAD pyrophosphatase was found in a potato extract. Then it may be said that there are in plants two isodynamic FAD pyrophosphatase as in animals.

The FAD hydrolysis by this enzyme proceeded specifically at the pyrophosphate linkage just as in the case of diphenyl pyrophosphate by diaryl pyrophosphatase. Since FMN and adenylic acid were identified as the products, the formation of any intermediate such as flavin pyrophosphate or flavin pyrophospho-ribose could be excluded.

SUMMARY

Diphenyl pyrophosphate is hydrolysed by diaryl pyrophosphatase into two moles of monophenyl phosphate. This enzyme is inactive to flavin adenine dinucleotide, which is in turn split by nucleotide pyrophosphatase to produce flavin monophosphate and adenylic acid. For either diaryl pyrophosphatase or nucleotide pyrophosphatase there are two isodynamic, acid and alkaline, enzymes. The acid FAD pyrophosphatase is found in potato extract and the alkaline enzyme in muscle autolysate. Acid diaryl pyrophosphatase contained in potato extract can be obtained free from isodynamic alkaline enzyme, whereas the two isodynamic diaryl pyrophosphatases of the liver, though they could be made free from other phosphatases, are incapable at present, of being separated from each other.

This work was supported in part by a grant from the Ministry of Education, given to Prof. S. Akamatsu, the director of this department.

REFERENCES

(*1*) Kurata, K., *J. Biochem.*, **14**, 25 (1931)
(*2*) Kornberg, A., and Pricer, W. E., *J. Biol. Chem.*, **182**, 763 (1950)
(*3*) Akamatsu, S., *J. Biochem.*, **39**, 203 (1952)
(*4*) Neuberg, C., and Wagner, J., *Biochem. Z.*, **171**, 485 (1926)
(*5*) Yagi, K., *J. Biochem.*, **38**, 161 (1951)
(*6*) Warburg, O., and Christian, W., *Biochem. Z.*, **298**, 150 (1938)
(*7*) Matsuzaki, K., *Vitamin*, **6**, 725 (1953) (in Japanese).

The Journal of Biochemistry, Vol. 43, No. 5, 1956

STUDIES ON THE INCORPORATION OF S^{35}-SULFATE INTO CHARONINSULFURIC ACID BY CHARONIA LAMPAS

By SAKARU SUZUKI

(*From the Department of General Education, Nagoya University, Nagoya*)

(Received for publication, June 13, 1956)

Recently Egami and coworkers (*1*) reinvestigated a new type of glucan polysulfate (charoninsulfuric acid) which was isolated from the mucus of *Charonia lampas* (*Tritonium nodiferm*), and showed that a part of the glucan has a cellulose structure and the other part an amylose structure. It was found in this experiment that a close relation exists between the sulfur content of charoninsulfuric acid and the constitution of the glucan, i.e. the less the sulfur content the less the cellulose structure. This parallelism implies that the biological transformation of amylose to cellulose may be preceded or accompanied by the sulfation, and hence the sulfation of charoninsulfuric acid is of particular interest.

The problems concerning the biological sulfation of polysaccharides in different mammalian tissues have been dealt with by several workers. Boström and Månsson (*2*) demonstrated that S^{35}-sulfate is retained in slices of cartilage of calf, and the major portion of the labeled sulfate is present in chondroitin sulfate. Sato and coworkers (*3*) demonstrated a similar uptake of sulfate in heparin by the liver of rats.

According to our study (*4*) on the incorporation of S^{35}-sulfate into different organs of *Chalonia lampas*, remarkably high concentrations of S^{35} are recognized in the mucous gland. It thus seems most reasonable to assume that the biological sulfation of charoninsulfuric acid occurs in the mucous gland. The present *in vivo* and *in vitro* investigations were undertaken to determine whether or not S^{35}-sulfate enters into charoninsulfuric acid of the mucous gland.

EXPERIMENTAL

In Vivo Experiments—Six groups of *Charonia lampas*, each group comprising four *Charonia* weighing 120 to 160 g. (without shell), were injected intramuscularly with S^{35}-sulfate in 0.2 ml. of distilled water (5×10^6

c.p.m. per sq.cm. measured as benzidine sulfate at infinite thickness). After immersing the test animals in a sea of 20°, the respective groups were killed at different times ; the first group after 3 hours, the last one after 69 hours. The mucous glands were removed and immediately placed in absolute methanol. After 4 days these were defatted with acetone and dried *in vacuo*. Charoninsulfuric acid was obtained from this preparation of each group as follows: about 10 g. of the dried mucous gland was ground to a powder and extracted with 150 ml. of 1.7 per cent HCl at 40° for 40 minutes. The clear extract was neutralized with KOH to salt out potassium charoninsulfate, which was then dissolved in 1 per cent HCl and reprecipitated with an equal volume of ethanol. After repetition of this salting out and precipitation procedures, the precipitate was dissolved in 50 ml. of distilled water and the last traces of S^{35}-sulfate were removed by electrodialysis. The dissolved charoninsulfuric acid was then precipitated with ethanol, washed with absolute ethanol and ether and dried *in vacuo*. About 200 mg. of each of the different preparations thus obtained were hydrolyzed with a mixture of conc. HNO_3 and HCl (1 :3) in a boiling water bath, and after evaporation the sulfate was precipitated as the benzidine salt from aqueous solutions. The benzidine sulfate was washed with 50 per cent and 95 per cent aqueous acetone successively, suspended in 2 ml. of absolute ethanol and then transferred to a small porcelain dish of a diameter of 2.5 cm. After drying, the radioactivity was measured by means of a Geiger-Müller counter. Each sample had a thickness of 17 mg. per sq.cm., which corresponded approximately to infinite thickness. Under such conditions the number of counts is linearly proportional to the activity. The results are expressed as counts per sq. cm. The error in measuring the radioactivity of the sample amounted to 8 per cent.

The possibility that the samples of charoninsulfuric acid obtained under this experiment were contaminated with free S^{35}-sulfate need not be considered because of the following fact: 200 mg. of pure charoninsulfuric acid (S=15 per cent) and S^{35}-sodium sulfate (5×10^6 c.p.m.) dissolved in 150 ml. of 1.7 per cent HCl were treated as above ; it was then found through the measurement of the radioactivity that the charoninsulfuric acid thus obtained was free from the labeled sulfate.

In Vitro Experiments—The mucous gland of newly killed *Charonia* was sliced to a thickness of approximately 0.5 mm. with a safety razor blade. About 35 g. each of the slices, which contain large amounts

of charoninsulfuric acid, were quickly transferred to three vessels containing 100 ml. of sea water as the basic medium. The vessels were shaken in a thermostat of 30°. One of the samples (No. I) was taken out and boiled for 20 minutes, cooled, and returned to the thremostat. When the temperature of this sample was 30°, S^{35}-sodium sulfate (3×10^6 c.p.m. per sq. cm.) in 1 ml. of distilled water were added to each sample and the reaction was allowed to proceed at 30°. Samples II and III were removed after 4 and 8 hours respectively, and boiled for 20 minutes. Each suspension was acidified with 5 ml. of conc. HCl, and charoninsulfuric acid was extracted as described in the preceding experiment. About 200 mg. each of the charoninsulfuric acid samples I to III were hydrolyzed with a mixture of conc. HNO_3 and HCl, and the sulfate was precipitated as the benzidine salt for the determination of the radioactivity.

RESULTS

The results of the sulfur and nitrogen analyses are given in Table I. The sulfur values, being relatively high, indicate that it must be

Table I

Analytical Figures for Different Samples of Charoninsulfuric Acid

Sample No.	Per cent of dry substance	
	S	N
In vivo experiments		
1	13.60	0.32
2	14.20	0.30
3	14.55	0.28
4	14.40	0.36
5	13.55	0.32
6	13.45	0.38
In vitro experiments		
I	15.00	0.28
II	14.80	0.24
III	14.80	0.22

excluded in this paper to make discussions on the charoninsulfuric acid with a low sulfur content. The nitrogen values indicate that small amounts of protein are present in all samples; but this contamination need not be taken into consideration, since the amounts of sulfur-con-

taining amino-acids are negligible.

In Fig. 1 the radioactivities measured in the *in vivo* experiments are plotted against time in hours after injection. As can be seen, the curve shows an increase in the radioactivity from 50 c.p.m. per sq. cm. after 3 hours to 825 c.p.m. after 41 hours and then a slow increase to 857 c.p.m. after 69 hours. A decrease in the radioactivity, *i.e.* elimination of labeled sulfur from the charoninsulfuric acid, could not be observed, since *Charonia* could not be kept alive beyond 3 days after injection.

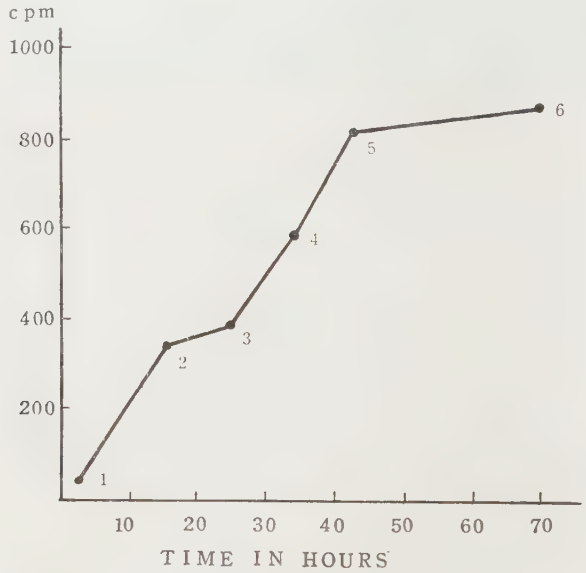


FIG. 1. Uptake of radioactive sulfate in charoninsulfuric acid of mucous gland.

Counts per minute per sq. cm. as found in measuring radioactivity plotted against time in hours.

The results of radioactivity measurements in the experiment performed with slices are shown in Table II. Even when no substrates have been added, slight uptake of S^{35}-sulfate was observed after 8 hours, while there was no significant uptake of S^{35} if the slice was boiled prior to the addition of the isotope. As the amounts of S^{35} fixed within 8 hours

TABLE II

Uptake of Radioactive Sulfate into Charoninsulfuric Acid by Slice of Mucous Gland

Sample No.	Time (*hours*)	Radioactivity of charoninsulfuric acid (*c.p.m. per sq.cm.*)
I (boiled)	8	40
II	4	78
III	8	135

were small, a clear-cut result regarding the effect of several inhibitors has not been obtained. It was impossible to incubate the reaction mixtures more than 8 hours owing to the putrefaction of the slices. Additions of 0.02 *M* glucose, 0.03 *M* phosphate or 0.5 per cent charoninsulfuric acids with different sulfur contents (S—2,5 and 15 per cent) did not accelerate the reaction.

DISCUSSION

From the consideration of these results and those obtained with autoradiography (*4*), it seems most reasonable to conclude that biological sulfation of charoninsulfuric acid occurs in the mucous gland, and that the sulfate ion in sea water is a natural source of the sulfate group of charoninsulfuric acid.

In the studies on the conjugation of sulfate with phenols, it has been demonstrated by several workers (*5–6*) that the preliminary step of the conjugation is an enzymatic activation of inorganic sulfate by ATP. Recently Hilz and Lipmann (*7*) have suggested that the active sulfate corresponds to a mixed anhydride between sulfate and phosphate, the phosphate being most likely linked to adenosine. Their suggestion must be useful for the studies on the biological sulfation of polysaccharides.

SUMMARY

1. The uptake of S^{35}-sulfate into the charoninsulfuric acid of the mucous gland of *Charonia lampas* has been studied by *in vivo* and *in vitro* experiments.

2. An increase in the radioactivity of charoninsulfuric acid was

demonstrated during 69 hours after intramuscular injection of S^{35}-sulfate.

3. Sodium sulfate labeled with S^{35} has been found to be taken up by the slice of the mucous gland and built into charoninsulfuric acid. In the boiled mucous gland no similar uptake occurs.

The author thanks Prof. Fujio Egami for his interest and encouragement. A part of the experiments was executed in the Marine Biological Laboratory of Nagoya University.

REFERENCES

(1) Egami, F., Asahi, T., Takahasi, N., Suzuki, S., Shikata, S., and Nisizawa, K., *Bull. Chem. Soc. Japan*, **28**, 685 (1955)
(2) Boström, H., and Månsson, B., *J. Biol. Chem.*, **196**, 483 (1952)
(3) Sato, T., Fukuyama, T., Suzuki, T., and Yoshikawa, H., *J. Japan. Biochem. Soc.*, **27**, 672 (1956)
(4) Suzuki, S., and Ōgi, K., *J. Biochem.*, **43**, 697 (1956)
(5) Bernstein, S., and McGilvery, R. W., *J. Biol. Chem.*, **198**, 195 (1952)
(6) De Meio, R. H., Wizerkaniuk, M., and Schreibman, J., *J. Biol. Chem.*, **213**, 439 (1955)
(7) Hilz, H., and Lipmann, F., *Proc. Natl. Acad. Sci.*, **41**, 880 (1955)

The Journal of Biochemistry, Vol. 43, No. 5, 1956

AUTORADIOGRAPHIC STUDY ON THE INCORPORATION OF S^{35}-SULFATE INTO MUCOUS GLAND OF CHARONIA LAMPAS

By SAKARU SUZUKI and KEN-ICHI ŌGI

(*From the Department of General Education, Nagoya University, Nagoya*)

(Received for publication, June 13, 1956)

In a previous investigation (*1*) on the incorporation of sulfate into the mucous gland of *Charonia lampas* (*Tritonium nodiferm*), we demonstrated that the major part of the fixed S^{35}-sulfate was found in the sulfate group of charoninsulfuric acid. However, nothing has so far been investigated about the relation between the histological feature of the mucous gland and its function in the biological sulfation.

The present investigation was undertaken in order to elucidate the role of the mucous gland in the biological sulfation of charoninsulfuric acid by means of autoradiography.

EXPERIMENTAL

Three groups of *Charonia lampas* (weighing about 150 g. without shell) were injected intramuscularly with 0.2 ml. of a solution containing 1.0 millicurie of S^{35}-sodium sulfate (carrier-free). Then all of the test animals were kept in a sea at 20° for varying periods (3, 45 and 69 hours) after the injection; the liver and mucous gland were removed and immediately placed in absolute methanol for 5 days. After treatment with isopropanol, paraffin embedding and sectioning in 15-μ thickness sections were made by the usual method. The sections were mounted on glass-slides and deparaffinized with xylene. One series of these preparations was cove red with celloidin and then with a stripping film by lifting the slide underneath the film (Fuji autoradiographic plate for stripping method) floating in 1 per cent glycerol solution. After exposure for 14 days at 5°, development was undertaken in the following solution at 20° for 3 minutes: distilled water, 1 litre; metol, 4 g.; anhydrous sodium sulfite, 60 g.; hydroquinone, 10 g.; anhydroussodium carbonate, 45 g.; potassium bromide, 2.5 g. After fixing and washing, the slides with the film were dried under the fan. Another series of the preparations was pressed directly against Fuji autoradiographic plates in iron screw presses for contact autoradiography. After exposure for 14 days, the films and sections were separated, and the films were developed, fixed and washed in the same manner. The latter (*i.e.*, the sections) were stained by Bauer's method (*2*) for polysaccharide location, and by Feulgen's method (*3*) for desoxy-

ribonucleic acid location, and compared with the autoradiographs.

RESULTS

The mucous gland of *Charonia lampas* is composed of the epithelial layer, connective tissue with folds and palisade of cylindrical mucous cells covering it, as shown in Fig. 1 and Fig. 2: usually the mucous cells contained large amounts of mucus (mucoprotein of charoninsulfuric acid) produced in it. As shown in Fig. 3, the connective tissues are

FIG. 1

FIG. 2

FIG. 3

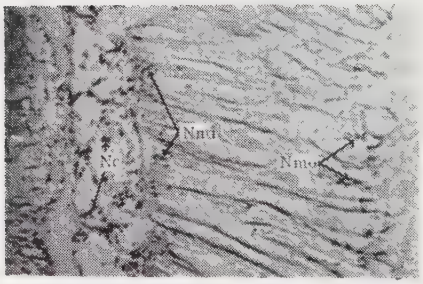


FIG. 1. Cross section of mucous gland stained with Bauer's method. (×4).

FIG. 2. Detail of Fig. 1.

E: epithelium, C: connective tissue, M: mucous cells. (×7)

FIG. 3. Microphotograph of section of mucous gland treated with Feulgen's nucleal reaction.

(Detail from portion indicated by boundary line in Fig. 2.) Arrows point to nuclei. (×75)

Nc: nuclei of cells of connective tissue.

Nmi: nuclei of inner mucous cells.

Nmo: nuclei of outer mucous cells.

FIG. 4

FIG. 5

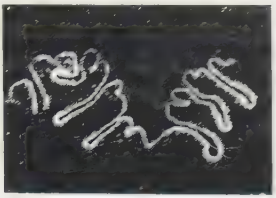


FIG. 4. Snapping from autoradiograph showing high S^{35}-uptake in mucous cells along connective tissue, 69 hours after injection of S^{35}-sulfate. × 1

FIG. 5. Contact autoradiograph of mucous gland (total), 69 hours after injection. × 4

surrounded by somewhat indistinct paris of mucous cells, the two cells being connected longitudinally. Feulgen's nucleal reaction revealed that innumerable granules, presumably nuclei, were present, and that these were located in areas nearest to the center connective tissue in the case of the inner cells, and in areas most apart from the connective tissue in the case of the outer cells. As apparent from the autoradiographs of Figs. 4 and 5, radioactive sulfur was mostly accumulated around the nuclei of the inner cells. No difference was observed between the autoradiograph of 45 hours and that of 69 hours after injection; the preparation obtained 3 hours after injection, however, failed to give blackening of the film.

On the other hand, all of the sections of liver failed to give any blackening of the films, irrespective of whether times of exposure and development were prolonged or not.

DISCUSSION

From a consideration of these results and those described in the previous paper in this series [7], it seems most reasonable to conclude that the biological sulfation of charoninsulfuric acid occurs near the nuclei of the inside mucous cells.

Odeblad and Boström [4] showed by means of autoradiography that S^{35}-sulfate is fixed in the liver of rats and rabbits 48 hours

after the injection. In contrast with their observations, we have been unable to catch any radioactive substances in the liver of *Charonia* by means of autoradiography.

The identification of radioactive substances other than charonin-sulfuric acid existing in the mucous cells as well as the elucidation of the mechanism of biological sulfation of polysaccharides must await further experiments.

SUMMARY

The incorporation of S^{35}-sulfate into the mucous gland of *Charonia lampas* was studied by means of autoradiography.

A particularly high uptake of S^{35} is noted in a layer bordering with the connective tissue in mucous cells. It is shown with Feulgen's nucleal reaction that numerous granules regarded as nuclei are distributed in the layer.

Sections of the liver, obtained 3, 45 and 69 hours after the injection of S^{35}-sulfate, give no blackening of the films in contrast with mammalian livers.

We thank Prof. Fujio Egami for his interest and encouragement. A part of the experiments was executed in the Marine Biological Laboratory of Nagoya University.

REFERENCES

(1) Suzuki, S., *J. Biochem.*, **43**, 691 (1956)
(2) Bauer, H., *Z. mikr. -anat. Forsch.*, **33**, 143 (1933)
(3) Feulgen, R., and Rossenbeck, H., *Z. physiol. Chem.*, **135**, 203 (1924)
(4) Odeblad, E., and Boström, H., *Acta Path. Microbiol. Scand.*, **31**, 339 (1952)

The Journal of Biochemistry, Vol. 43, No. 5, 1956

PHYSIOLOGICAL CHEMISTRY OF THE HARD TISSUES

By YOSOJI ITO, SUSUMU TSURUFUJI, YOSIO HIRAMATSU and MIKITO CHIBA

(From the Institute of Physiological Chemistry, Department of Pharmacy, Faculty of Medicine, University of Tokyo, Tokyo)

(Received for publication, June 20, 1956)

An isotopic tracer method using Ca^{45} or P^{32} have made great contributions to the study of physiology and biochemistry of the calcified tissues. Above all, remarkably rapid incorporations of Ca^{45} or inorganic P^{32}-phosphate into the mineral crystals of the calcified tissues from surrounding medium, both in vivo and in vitro, has surprised many workers. It is generally accepted that such a rapid uptake of radioisotopes results largely from physicochemical interchange of these ions between solid phase (mineral crystals) and liquid phase (body fluid). Hence some workers are disappointed for the use of radioisotope as an indicator of bone metabolism (*1*, *2*).

The rate of the incorporation of radioisotopes into calcified tissues *in vivo* was estimated by some workers (*3*, *4*), but these reports pay little attention to the distinction between the uptake of radioisotope due to physicochemical exchange and physiological addition which results from the formation of newly calcified tissues. In the skeletal tissues, even in the adult, resorption and new formation of the tissue coexist in a microscopical scale. Because of such complexity of reconstruction processes in bone tissues, it is difficult to distinguish the incorporation of Ca^{45} due to the physicochmical exchange from that due to the physiological accretion of bone salt (*5*). On the contrary, the incisor of the rodent grows permanently all through the life time. There occurs no such complex reconstruction processes as in the bone. Problems to be considered in the incisor are only physicochemical exchange, formation of newly calcified tissues, and mechanical attrition of the tip. Moreover, the amount of new formation should be equal to that of attrition in mature animals so that the incisor of the rodent is a profitable material for the quantitative study on the incorporation of the radioisotopes into the calcified tissues.

This experiment was attempted mainly to estimate the ratio of

physicochemical exchange to physiological accretion in the incorporation process of radioactive calcium into the incisor of the rat.

METHOD

Experimental Animals—Groups of litter mates of male albino rats were used and placed on a suitable diet.

Assay of Calcium and Ca^{45} in the Serum—Blood was drawn by cutting the common carotid artery. Total calcium level of serum was measured by the slightly modified method of Clark and Collip (*6*). Radioactivity assay of serum Ca^{45} was carried out as follows, with the exception of that described later: 0.5 ml. of saturated ammonium oxalate solution was added immediately to 1 ml. of the serum placed in a shallow stainless steel sampling dish. This was mixed thoroughly, stood overnight, and centrifuged. Supernatant fluid was discarded with a hooked capillary pipette, and resultant calcium oxalate precipitate, after washing once with 2 per cent NH_4OH, was dried mildly under an electric infrared heater lamp. Radioactivity was measured by means of a thin mica-window G-M counter.

Measurement of the Incisor—Rats were all sacrificed by cutting the carotid artery. Bilateral lower incisors were removed, and adherent soft tissue other than dental pulp was wiped off carefully with a filter paper. The cleaned incisor was calcinated at 600–700° in a porcelain crucible of B-OO type in an electric muffle furnace. Resultant ash was weighed, dissolved in 0.5 ml. of 20 per cent HCl, evaporated to dryness under an electric infrared heater lamp, and dissolved again in 2 ml. of 1 *N* HCl. Then calcium was precipitated as the oxalate and centrifuged in the crucible. The precipitate was dried at 100°. Thus radioactivity of labeled Ca^{45} $(COO)_2$ H_2O in the crucible[1] was assayed with a thin mica-window G-M counter. Quantitative measurement of the incisor calcium was also carried out gravimetrically with the same sample. All measurements of radioactivity were corrected for self-absorption, background, radioactive decay, geometrical condition, and for changes in counter efficiency.

RESULTS

Constancy of the Specific Activity of Serum Calcium—Single administration of the solution of radiocalcium or inorganic radiophosphate causes an abrupt, but temporary, rise of radioactivity in the serum, whether it is given orally (*4*), subcutaneously (*7*), intravenously (*7*), or intraperitoneally (*2*, *8*).

1) The porcelain crucible has a little radioactivity, about one- third the natural backgound. It may come from natural clay material and mainly consists of moderately hard beta-ray. The radioactivity of the crucible was treated as a part of the background radiation.

Therefore, repeated administrations are necessary to keep the specific activity of serum calcium at a constant level. Through some preliminary investigations to determine relevant doses and intervals of Ca^{45} administration, a certain appropriate procedure was found to give a constant specific activity of serum calcium.

Each of 7 rats, weighing about 190 g., was injected with $Ca^{45}Cl_2$ solution intraperitoneally by the "AGLA" micrometer syringe[2] as follows: 4 μC. at the first, followed by 10 injections of 1 μC. at 30 minutes intervals all through the experiment. Because of the high specific activity[3] of the injected calcium solution, total injected dose of 14 μC. of Ca^{45} accompanied with 0.47 mg. of natural Ca^{40} for each rat is considered physiologically carrier-free (*8*).

Repeated radioactivity assay of rat serum was carried out. About 0.3 ml. of the blood sample was drawn from the tail vein of each rat and centrifuged. 0. 1 ml. of the resultant serum was dried in a stainless steel sampling dish[4] as a thin film (less than 3 mg. per cm.^{2}) and assayed with the G-M counter. Constancy of the radioactivity of the serum was accomplished as seen in Fig. 1.

Uptake of Labeled Calcium by the Incisor Teeth on the Constant Specific Activity of the Serum Calcium—As in the foregoing cases 19 rats weighing about 190 g. were given repeated intraperitoneal injections of Ca^{45} in order to maintain the specific activity of serum calcium at a constant level. The rats were sacrificed in 1 to 4 animals at intervals of 30 minutes from 30 to 270 minutes after the first injection. Specific activity of serum calcium and radioactivities of the lower incisor teeth were determined.

Specific activity of the calcium incorporated into the incisor tooth is assumed to be equal to that of serum calcium with slight, practically negligible retardation. Based on this assumption, if specific activity of serum calcium is kept constant, total calcium uptake by the incisor, which includes both physicochemical exchange of calcium between incisor mineral and blood serum (interstitial fluid) and physiological addition of calcium due to the new tissue formation, can be computed from the following equation.

$$\text{Incorporated Ca (mg.)} = [\text{radioactivity of incisor (c.p.m.)}] \div [\text{specific activity of serum calcium (c.p.m. per mg.)}] \cdots\cdots (1)$$

2) Burroughs Wellcome Co., London

3) 30 μC. per mg. Ca in 0.4 ml.

4) Scientific Research Institute, Ltd., Tokyo, Japan.

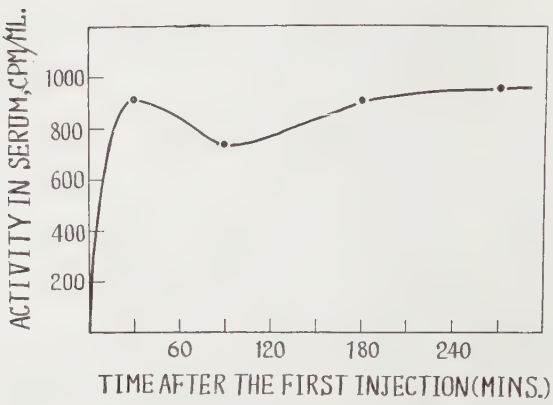


Fig. 1. Radioactivity of serum calcium of rat with repeated intraperitoneal injections of physiologically carrier-free $Ca^{45}Cl_2$ solution. Each point consists of seven measurements exclusive of the first point that consists of four assays. Each rat was injected 4, 1, 1, 1, . . . μC. of Ca^{45} at intervals of thirty minutes.

The rate of incorporation of calcium into the incisors computed from Eq. (1) is shown in Fig. 2. With the passage of time, the amount of incorporated calcium increases rectilinearly. The values plotted against time are the amount of incorporated calcium computed from Eq. (1) and each point is the average of the values obtained in lower bilateral incisors of indvidual animals.

Rate of Physiological Deposition of Calcium to Permanently Growing Incisor Tooth—It is difficult, as observed diagramatically in Fig. 3, to measure directly the amount of newly formed dental tissue, but the amount of the formation of new dental tissue is equal to that of attrition of the tip, which was measured with ease as follows:

Twenty normal rats weighing about 180 g. were given intraperitoneal injection of 0.3 ml. of 4 per cent sodium alizarinesulfonate solution. They were sacrificed on 17th day after injection of the dye, and bilateral lower incisor teeth were removed. Though apical and medial parts of the teeth were stained purple by the alizarin dye, the basal part, which was formed after the injection, remained colorless. The length of the colorless portion, which shows the rate of growth of the incisor tooth, was measured under a microscope. The average of the values measured along lingual side of the arc of lower incisor was 0.407 ± 0.014 mm.[5]

5) Mean $\pm$ standard deviation.

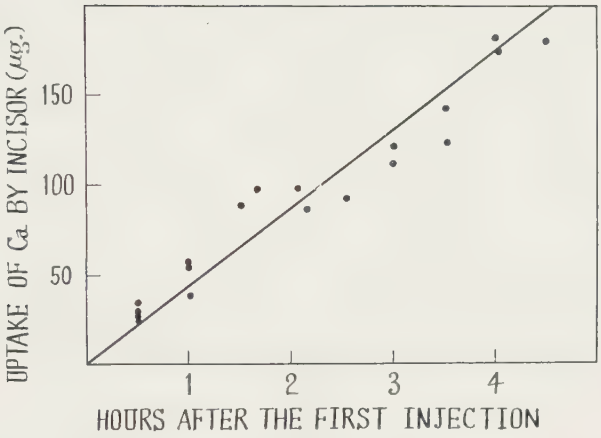


Fig. 2. Total uptake of labeled calcium by rat incisors through both new dental tissue formation and physicochemical exchange determined on the constant specific activity of serum calcium.

Each rat was injected with physiologically carrier-free $Ca^{45}Cl_2$ solution intraperitoneally. Administration of 4 μC. at the first was followed by repeated injections of each 1 μC. at thirty minutes intervals in order to maintain constant specific activity of serum calcium.

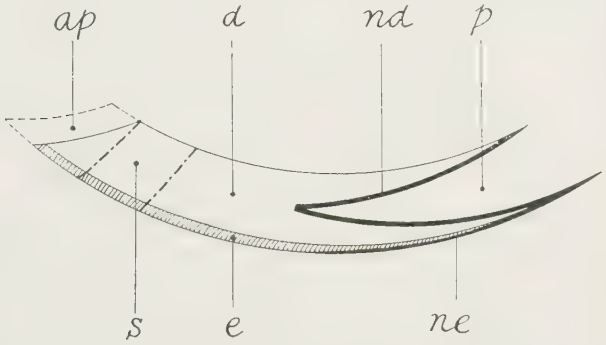


Fig. 3. Lower incisor of the rat (diagramatical).

d: Dentin. e: Enamel. p: Pulp. ne: Layer of newly formed enamel. nd: Layer of newly formed dentin. ap: Defaced apical part. s: Section to be assigned for the analysis of calcium.

per day: *i.e.*, 0.0170 ± 0.0006 mm.[5] per hour.

Apical parts of the incisors which do not contain any soft tissue

or dental pulp, were cut off as shown in Fig. 3. The length of these parts was measured and the quantity of calcium was analysed. The average rate of wearing of each incisor during the experiment can be calculated from the rate of growth and the amount of calcium against unit length of apical part as follows:

$$\text{Average rate of wearing of incisal calcium (mg. per hour)} = \frac{[\text{amount of Ca in cut part (mg.)}] \times [\text{rate of growth (mm. per hour)}]}{[\text{length of the cut part (mm.)}]} \quad \cdots (2)$$

The calculation were made on the individual lower incisors. The mean value for 40 incisors (20 rats) was 25.8 ± 1.1[5] micrograms per hour (0.62 ± 0.026 mg.[5] per day). This value should correspond to the rate of the deposition of calcium by the incisal tissue formation.

DISCUSSION

Labeled calcium is incorporated into the incisal tissues both by physicochemical and physiological means. If specific activity of surrounding body fluid is constant, uptake of Ca^{45} by physicochemical exchange between dental mineral and body fluid will gradually approach the maximum state, as shown in Fig. 4 A. In other words, when all the exchangeable calcium in dental tissues attains the state of equilibrium with serum Ca^{45}, total amount of Ca^{45} in the incisor will no longer increase by a physicochemical process. This conception is consistent with the data of in vitro experiment with pulverized fresh human teeth by Underwood and Hodge (*9*).

Amount of Ca^{45} incorporated into the incisor with new tissue formation will increase at a constant rate as shown in Fig. 4 B. *In vivo*, labeled calcium is incorporated into permanently growing incisor tooth through two processes, physicochemical exchange and physiological deposition. Therefore, time curve of overall uptake of labeled calcium by the incisor can be plotted as shown in Fig. 4 C. The rate of incorporation of labeled calcium will be the greatest in the initial portion of the curve. With passage of time, slope of the curve will gradually decrease, and finally takes the same value as the rate of physiological deposition attributable to the formation of new tissues.

As the relation between time and overall uptake of labeled calcium is rectilinear in the experiment of Fig. 2, it may be concluded that the rate of the incorporation of Ca^{45} by physicochemical exchange is not apt to decrease in the course of experimental period of 270 minutes.

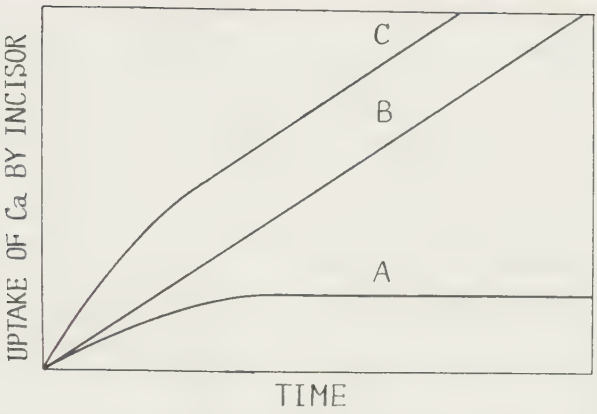


FIG. 4. Mechanism of deposition of labeled calcium into continuously growing incisor tooth.

A: Deposition by physicochemical exchange. B: Deposition by pysiological accretion. C: Total deposition attributable to both processes.

Therefore, the slope of the line of Fig. 2 shows an average initial rate of the overall incorporation of calcium into the lower incisor tooth, *i.e.* 44 micrograms per hour. This value is the total of physiological and physicochemical incorporation. The rate of physiological incorporation of calcium due to tissue formation has been shown in the present study, to be 25.8 micrograms per hour. Therefore, the amount of physicochemical exchange may be obtained by subtracting the amount of calcium of newly formed tissue from the total amount of incorporated calcium,

$$44-25.8=18.2 \text{ (micrograms per hour)}$$

Thus, it is concluded that the ratio of physicochemical exchange to physiological deposition is $18.2:25.8=4:6$.

Now it is necessary to consider the possibility of an introduction of any unreliable factor into such a conclusion. In histological studies, it has been pointed out that the incisors of some rodents seem to show periodical excitation in the rate of calcification, placidity in the day time and excitement at night (*10*, *11*, *12*). In the experiment of Fig. 2 the rate of incorporation of labeled calcium was measured in the day time. Therefore, whether any difference would exist in the uptake of calcum by the incsor of the rat between day and night was examined.

Such a histological periodicity is characteristic in the rabbit but is relatively ambiguous in the albino rat. The data from the experiment are tabulated in Table I. To sustain the Ca^{45} level of the blood serum constant the rats in the experiment of Figs. 1 and 2 were given 4, 1, 1, 1, 1, 1 . . . μC. of Ca^{45} solution at intervals of 30 minutes. Under such a condition, a slight fall is observed in specific activity of serum calcium 90 minutes after the first injection. Now the rats in Table I were administered intraperitoneal injections of 6, 2, 3, 2, 2, 1, 2, 1, 2, 1, 1, 1, and 1 μC. of $Ca^{45}Cl_2$ solutions at intervals of 30 minutes in order to correct such a temporary fall of serum Ca^{45} as seen in Fig. 1. No significant difference was observed in overall uptake of calcium by lower incisor between day and night during 6.5 hours' experimental period, as shown in the 6th column of Table I.

The average value of the rate of incorporation of calcium into the incisor in the said 6th column is 32.3 micrograms per hour. This value is less than the one (44 micrograms) in the experiment of Fig. 2.

However, it may be quite reasonable, since those animals in Table I are smaller in body weight than the animals in Fig. 2. The same phenomena is also shown in Table I. Though ages of the animals are equal, the litter No. 1 are significantly larger both in body weight (at 0.5 per cent level of significance) and in overall calcium uptake (at 0.5 per cent level of significance) than the litter No. 2, as seen in the 4th and 6th columns. A sample correlation coefficient between body weight and overall calcium uptake computed from the 4th and 6th columns is 0.96, is highly significant (at 0.1 per cent level of significance). Fiducial intervals of correlation coefficient in population, ρ, is obtained as follows :

$$0.765 < \rho < 0.994 \text{ (at 1 per cent level of significance)}$$

and indicates highly significant correlation between body weight and overall calcium uptake by incisor teeth. No difference in ash weight of the incisor is observed between these two groups. It may be suggested, therefore, though no direct evidence is present that the rate of the dental tissue formation, accordingly the rate of the attrition also, would be larger in a larger body weight group.

SUMMARY

1. Within 4.5 hours' experimental period, rectilinear relationship is observed between time and overall uptake of calcium including physicochemical exhange and physiological accretion by the permanently grow-

Table I

Comparison of Total Uptake of Calcium at Night with That in the Day on the Constant Specific Activity of Serum Calcium of the Rat

Each rat was injected with physiologically carrier-free $Ca^{45}Cl_2$ solution intraperitoneally. Administration of 6 μC. at the first was followed by the injections of 2, 3, 2, 2, 1, 2, 1, 2, 1, 1, 1, 1 and 1 μC. at intervals of thirty minutes in order to maintain constant specific activity of serum calcium.

Group	Litter No.	Animal No.	Body weight	Ash weight of lower incisor	Uptake of Ca by lower incisor during 6.5 hrs.
			g.	*mg.*	*μg.*
Expt. at night	I	23	159	43.6	238
		24	154	46.7	234
		25	145	45.3	254
	II	26	112	45.9	182
		27	112	42.7	166
		28	107	43.6	158
	Mean		132	44.6	205
Expt. in the day-time	I	29	135	42.2	224
		30	159	46.5	242
	II	32	113	43.6	183
		33	129	44.6	214
	Mean		134	44.2	216

ing lower incisor of the rat.

2. No difference is observed in overall uptake of calcium by the incisor between day and night.

3. In the initial period after the administration of radioactive calcium, physicochemical exchange and physiological deposition form, respectively, 40 and 60 per cent of the overall incorporation of Ca^{45} into the lower incisor of the rat.

4. Correlation between overall uptake of calcium by the incisor and body weight of the animals are discussed.

REFERENCES

(*1*) Sacks, J., Radioactive tracers in biochemistry and physiology, McGraw

Hill Co., New York, p. 284 (1953)
(2) Neuman, W. F., and Riley, R. F., *J. Biol. Chem.*, **168**, 545 (1947)
(3) Hevesy, G., Levi, H. B., and Rebbe, O. H., *Biochem. J.*, **34**, 532 (1940)
(4) Harrison, H. E., and Harrison, H. C., *J. Biol. Chem.*, **185**, 857 (1950)
(5) Neuman, W. F., and Weikel, J. H., Jr., *Annal. New York Acad. Sci.*, **60**, 685 (1955)
(6) Clark, E. P., and Collip, J. B., *J. Biol. Chem.*, **63**, 461 (1925)
(7) Hevesy, G., Radioactive indicators, Interscience Publishers, Inc., New York, p. 100 (1948)
(8) Talmage, R. V., Kraintz, F. W., and Kraintz, L., *Proc. Soc. Exptl. Biol. Med.*, **80**, 553 (1952)
(9) Underwood, E. E., and Hodge, H. C., *J. Dent. Research*, **31**, 64 (1952)
(10) Schour, I., and Stedman, S. R., *Anatom. Record*, **63**, 325 (1935)
(11) Schour, I., and Hoffman, M. M., *J. Dent. Research*, **18**, 91 (1939)
(12) Okada, M., *Folia Pharmacol. Japonica*, **25**, 122 (1937) (in Japanese)

The Journal of Biochemistry, Vol. 43, No. 5, 1956

MICROBIOLOGICAL DEGRADATION OF BILE ACIDS

V. ON A PRINCIPAL INTERMEDIATE IN THE BREAKDOWN OF CHOLIC ACID BY STREPTOMYCES

By YASUO SABURI*, SHOHEI HAYAKAWA**, TAKAO FUJII** and IKURO AKAEDA**

(*First Bacteriological Division, The Institute for Infectious Disease, The University of Tokyo, Tokyo, and **Department of Biochemistry, Okayama University Medical School, Okayama)

(Received for publication, July 5, 1956)

A microbiological transformation of cholic acid into a bile acid containing α, β-unsaturated ketone group was reported independently by Hayakawa (*1–3*) and Halperin *et al.* (*4*). Halperin *et al.* have demonstrated that a degradation product of cholic acid by *Nocardia sp.* was probably 3-keto-Δ^4-cholanic acid derivative and this reaction was catalyzed by DPN-linked dehydrogenase. Hayakawa *et al.* (*1–3, 5*), on the other hand, have separated a new unsaturated C-22 acid of m.p. 280–282° (decomp.) as an oxidation product of cholic acid by *S. gelaticus* 1164, and this C-22 acid has been defined to be 7α-hydroxy-3,12-diketo-Δ^4-bisnorcholenic acid. Though the exact position of one double bond remained to be determined, this observation was the first to demonstrate a β-oxidation of the side chain of cholic acid by microorganisms. In a previous paper (*6*) of this series, seven species of *Streptomyces* which utilize cholic acid as the sole source of carbon have been selected and identified. It then became of interest to expand our studies to the metabolism of various bile acids by these species. In the present communication, the following studies were performed:

1) The best condition of the culture media for the formation of the C-22 acid by *S. gelaticus* 1164; 2) The spectrophotometric analyses of the degradation products of cholic acid by the seven species of *Streptomyces*; 3) Effect of several bile acids other than cholic acid on the growth of the seven species of *Streptomyces*.

EXPERIMENTAL AND RESULTS

Effect of Cholate Concentration and Incubation Time on the Formation of the C-22 Acid by S. gelaticus 1164—Into the basal mineral medium ($(NH_4)_2$-

SO_4 2.0 g., K_2HPO_4 1.0 g., $MgSO_4 \cdot 7H_2O$ 0.5 g., $FeCl_3 \cdot 6H_2O$ 0.01 g., distilled water 1000 ml.) sodium cholate was added in final concentrations of 0.05, 0.1, 0.2, 0.3, 0.4 and 0.8 g. per dl., and their pH were adjusted to 7.2. Hundred milliliters, each of these media placed in 500 ml. Sakaguchi's flask was autoclaved at 110° for 20 minutes. *S. gelaticus* 1164 from Czapeck's slant culture (three days' incubation at 27°) was inoculated with a platinic loop in these media. All the six flasks were shaken for 20 days at 27° on a reciprocal shaker (120 r.p.m., 5 cm. amplitude). After 3, 4, 5, . . . and 22 days' incubation, an aliquot of the culture was centrifuged at 3,000 r.p.m. for one minute. The optical density at 246 mμ of the supernatant fluid was measured by Beckman spectrophotometer, type DU II. The C-22 acid showed the absorption maximum at 240.3 mμ in alcohol and at 246 mμ in water within the pH range of 5 to 9, and the optical density of this compound, within the range of concentrations likely to be encountered, obeyed the Beer's law. Effect of the cholate concentration and incubation time on the formation of the C-22 acid is given in Table I. In order to prove the degradation of cholic acid and the production of the C-22 acid, Pettenkofer's test and acid precipitation test of the culture medium were performed in parallel with the measurement of ultraviolet absorption at 246 mμ throughout the incubation period. The data are shown in Fig. 1.

As seen in Table I and Fig. 1, 1) a rapid increase in optical density at 246 mμ was observed, when the cholate concentration was 0.2 g. per dl. or less, while at 0.3, 0.4 and 0.8 g. per dl. the optical density increased more slowly ; 2) the cholate concentration and incubation time had no effect on the absolute amount of the C-22 acid formed, and about 18.8 to 23.9 per cent of the original cholate was converted into the C-22 acid in all the flasks ; 3) the C-22 acid formed in these media was gradually degraded, especially at high cholate concentrations ; 4) about one half of the amount of the C-22 acid calculated from its molecular extinction coefficient was isolated as crystals. This fact suggests that the ultraviolet absorption maximum at 246 mμ is not only due to the C-22 acid but also to some unidentified α, β-unsaturated ketone derivatives formed from cholic acid in the culture medium ; 5) Pettenkofer's test and acid precipitation test disappeared in parallel with the formation of the C-22 acid ; 6) shaking culture shortened the incubation time for the formation of the C-22 acid in about one half of that of a stationary culture (*1–3*).

Spectrophotometric Analyses of the Degradation Products of Cholic Acid

TABLE I

Effect of Cholate Concentration and Incubation Time on the Formation of the C-22 Acid by S. gelaticus 1164

Cholate concn. (*g./dl.*)	Incubation time (*days*)							
	3	4	5	6	7	10	13	20
0.05	7	24	92 (21.2)	40	16	4	4	8
0.1	20	60	192 (21.2)	84	10	13	10	20
0.2	22	71	364 (20.9)	156	46	41	32	21
0.3	10	39	188	624 (23.9)	376	388	280	160
0.4	33	51	160	696 (20.0)	468	620	344	64
0.8	2	8	22	41	56	616	1310 (18.8)	1280

The amount of the C-22 acid formed is given γ/ml. and per cent of the C-22 acid formed to the original cholic acid added is given in parentheses.

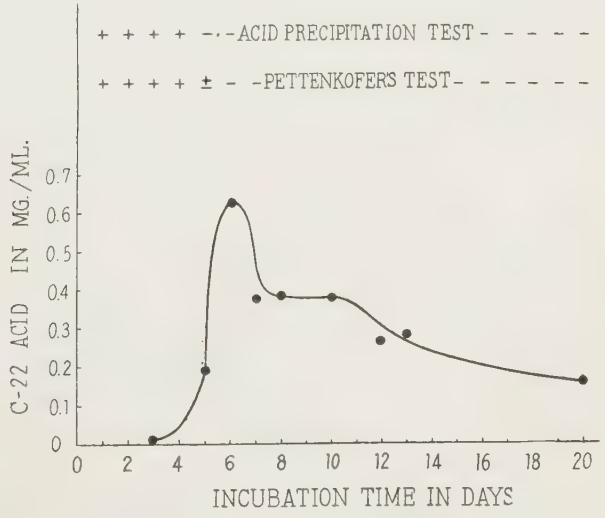


FIG. 1. Degradation of cholic acid and production of the C-22 acid by *S. gelaticus* 1164.

(Sodium cholate: 0.3 g./dl.).

by the Seven Species of Streptomyces—The degradation products of cholic acid by the seven *Streptomyces spp.* which were confirmed to utilize cholic acid as the sole source of carbon in the previous paper (*6*) were investigated by the following methods: Dulaney's mineral solution (*6*) containing 0.1 g. of sodium cholate per dl. was distributed in small test tubes, each receiving 2 ml., and autoclaved at 110° for 20 minutes. Each of the tubes was inoculated with one of the seven *Streptomyces spp.* After stationary incubation for 5 days at 27° in darkness, the culture fluid was centrifuged at 3,000 r.p.m. for one minute, and the resulting supernatant was divided into two parts after 40-fold dilution. The ultraviolet absorption spectra of the one part were measured. All of the seven *Streptomyces* tested gave an absorption peak at 246 mμ as shown in Fig. 2. To the aliquots of the other part was added separately a tenth volume of aqueous N NaOH or concentrated hydrochloric acid and the mixture was heated in a boiling water bath for ten minutes. After chilling, the ultraviolet spectra of each culture were measured. All of the seven *Streptomyces* tested gave an absorption peak at 290 mμ as shown in Figs. 3 and 4.

As controls, these *Streptomyces spp.* were cultured in a medium containing glucose (1.0 g. per dl.) in place of sodium cholate for ten days at 27°. The supernatants obtained according to the procedure described above showed no absorption peak at 246 mμ, but showed a peak at 270 mμ in the cases of *S. gelaticus* 1164, *S. flavogriseus*, *S. californicus* and *S. rubescens*. An absorption peak at 240 mμ was observed only for *S. halstedii*. Such evidences suggest that an absorption peak at 246 mμ is due to the degradation products of cholic acid in the culture medium. Also the shift of $\lambda_{max.}^{water}$ by alkali or acid from 246 mμ to 290 mμ suggests that all of the seven *Streptomyces* may degrade cholic acid through an intermediate having the same chromophore.

Utilization of Various Kinds of Bile Acids by these Seven Cholic Acid Utilizers—Autoclaved Dulaney's mineral solution (*6*) was used as a basal medium and to the solution were added sodium salts of eleven kinds of bile acid. The concentration of these bile salts was made up to 0.1 per cent and the resulting culture media were sterilized fractionally at 100°. These media were tubed in 1 ml. quantity in small test tubes and the seven cholic acid utilizers were inoculated from Czapeck's agar (7 days' incubation at 27°). The growth of *Streptomyces spp.* was examined for ten days. The results are shown in Table II.

The position and the number of hydroxyl group and the side chain

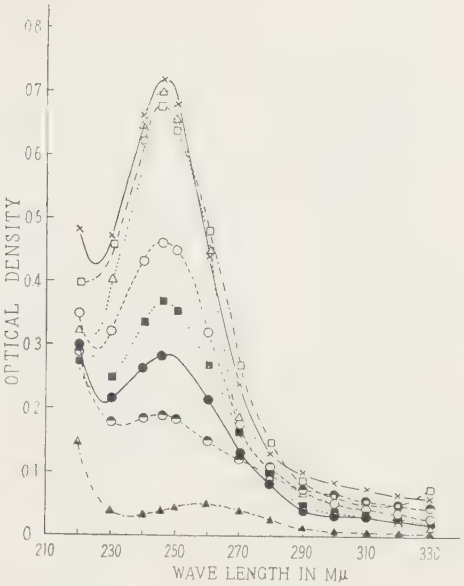


FIG. 2. Ultraviolet absorption spectra of the culture filtrates (diluted 1:40) obtained after incubation of cholic acid with the seven species of *Sreptomyces* for 5 days at 27°.

—×—, *S. scabies*; ···△···, *S. rubescens*; -·-□-·-, *S. halstedii*; --○--, *S. gelaticus* 1164; ···■···, *S. californicus*; —●—, *S. nitrosporeus*; ---●---, *S. flavogriseus*; -·-▲-·-, 40-fold diluted Dulaney's mineral solution only.

length of the various kinds of bile acids played an important role for the utilization of bile acid by *Streptomyces spp.* tested. Chenodesoxycholate and desoxycholate were considered to be toxic against all of the seven *Streptomyces* tested because they showed no growth even when cultivated in the medium cotaining glucose (1.0 g. per dl.), and homocholate and α-hyodesoxycholate against some of the species, but norcholate had no toxicity for all of the seven species.

DISCUSSION

Various microbiological transformations of steroids were reviewed by Peterson (*7*), but he did not describe in his review the conversion of bile acid by microbes. However, it has been demonstrated that *S.*

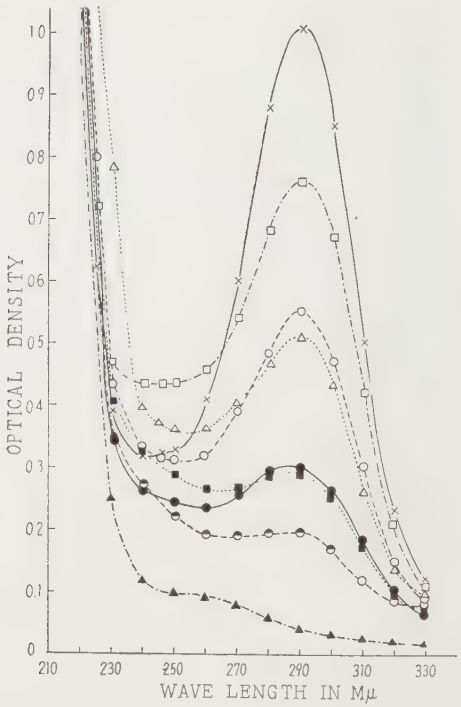


FIG. 3. Ultraviolet absorption spectra of the caustic alkali-treated culture filtrates (diluted 1:40) obtained after incubation of cholic acid with the seven species of *Streptomyces* for 5 days at 27°.
See footnotes in Fig. 2.

gelaticus 1164 is capable of converting cholic acid into a C-22 acid, namely, 7α-hydroxy-3,12-diketo-Δ^4-bisnorcholenic acid (*1–3,5*). The C-22 acid shows an ultraviolet absorption maximum at 246 mμ in water and is transformed by treating with caustic alkali or sulfuric acid into 3,12-diketo-$\Delta^{4,6}$-bisnorcholadienic acid (*5*) which has an absorption maximum at 290 mμ in water.

In the present study six species of *Streptomyces* were studied in this respect along with *S. gelaticus* 1164. Cultures of all of the seven cholic acid utilizers showed an absorption maximum at 246 mμ (Fig. 2) and their culture supernatants which were heated with caustic alkali or hydrochloric acid indicated an absorption maximum at 290 mμ (Figs. 3 and 4). These findings, therefore, indicate that the six cholic acid

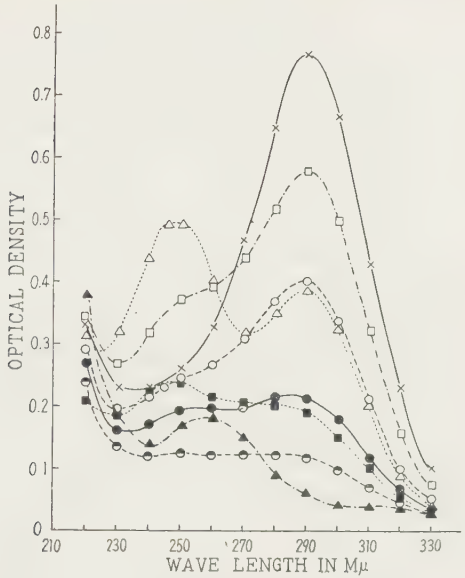


FIG. 4. Ultraviolet absorption spectra of the hydrochloric acid-treated culture filtrates (diluted 1:40) obtained after incubation of cholic acid with the seven species of *Streptomyces* for 5 days at 27°.
See footnotes in Fig. 2.

utilizers tested are able to degrade cholic acid to an unidentified derivative containing an α, β-unsaturated ketone grouping which is probably 7α-hydroxy-3,12-diketo-Δ^4-bisnorcholenic acid, and seems to afford a substance containing $\Delta^{4,6}$-3-ketone grouping by treating with alkali or acid.

Gallagher *et al.* (*8*) showed that the oxidation of cholic acid by chromic acid proceeds most easily at C_7, and least at C_3. This order has been suggested previously by the work of Kaziro *et al.* (*9*) and Hoehn *et al.* (*10*). Haslewood (*11*, *12*) likewise confirmed this finding. On the other hand, Schmidt *et al.* (*13*) stated that cholic acid added to cultures of a strain of *Alcaligenes faecalis* or *E. coli* isolated from human faeces, was converted into different ketocholanic acids among which they isolated 3,7,12-triketocholanic (dehydrocholic) acid, and further they (*14*, *15*) demonstrated that 3α,12α-dihydroxy-7-ketocholanic acid and 3α-hydroxy-7,12-diketocholanic acid were two of the intermediate derivatives formed during bacterial oxidation of

TABLE II

Utilization of Vavrious Kinds of Bile Acids by Seven Cholic Acid Utilizers

Bile acids (sodium salt)			Cholic acid utilizers						
Name	Position of OH gpoups	Side chain	*S. scabies*	*S. halstedii*	*S. rubescens*	*S. nitrosporeus*	*S. californicus*	*S. flavogriseus*	*S. gelaticus* 1164
Tauro-cholate	3,7,12	$-CH(CH_3)\cdot CH_2\cdot CH_2\cdot CO\cdot NH\cdot CH_2\cdot CH_2\cdot SO_3H$	5	5	4	5	5	5	5
Glyco-cholate	3,7,12	$-CH(CH_3)\cdot CH_2\cdot CH_2\cdot CO\cdot NH\cdot CH_2\cdot COOH$	4	5	4	4	4	4	4
Lithocholate	3	$-CH(CH_3)\cdot CH_2\cdot CH_2\cdot COOH$	6	–	6	–	5	7	7
α-Hyodesoxycholate	3,6	,,	–	–	–	–	–	–	–
Chenodesoxycholate	3,7	,,	–	–	–	–	–	–	–
Desoxycholate	3,12	,,	–	–	–	–	–	–	–
Cholate	3,7,12	,,	4	4	4	4	4	4	4
Dehydrocholate	—	,,	6	7	4	4	4	4	6
Bisnorcholate	3,7,12	$-CH(CH_3)\cdot COOH$	–	–	4	–	–	5	9
Norcholate	3,7,12	$-CH(CH_3)\cdot CH_2\cdot COOH$	–	–	–	–	–	–	–
Homocholate	3,7,12	$-CH(CH_3)\cdot CH_2\cdot CH_2\cdot CH_2\cdot COOH$	–	–	–	–	–	–	–

Figures refer to period necessary for positive growth (in days) and minus signs mean non-growth.

cholic acid to dehydrocholic acid. Recently, Hayakawa *et al.* (*16*) found that 3α,12α-dihydroxy-7-ketocholanic acid was formed when cholic acid was incubated with a culture of *E. coli*. These findings are of interest, for they show that microbiological oxidation follows the same path as chemical oxidation and affords the 3α,12α-dihydroxy-7-keto acid, the 3α-hydroxy-7,12-diketo acid, and finally dehydrocholic acid, namely, the order of oxidation of the hydroxyl groups in cholic acid is $C_7 > C_{12} > C_3$.

The formation of 7α-hydroxy-3,12-diketo-Δ^4-bisnorcholenic acid from cholic acid by *S. gelaticus* 1164 and the data as seen in Figs. 2, 3 and 4 may suggest two possible pathways in the microbial oxidation of

cholic acid.

1) Cholic acid → 3α,12α-Dihydroxy-7-ketocholanic acid → 3α-Hydroxy-7,12-diketocholanic acid → 3,7,12-Triketocholanic (Dehydrocholic) acid →

2) Cholic acid → 3α,7α-Dihydroxy-12-ketocholanic acid (or 7α,-12α-Dihydroxy-3-ketocholanic acid) → 7α-Hydroxy-3,12-diketocholanic acid . . . → (?) . . . → 7α-Hydroxy-3,12-diketo-Δ^4-bisnorcholenic acid →

The first pathway is the above-described *Alcaligenes faecalis* or *E. coli* type and is the same as observed in the chemical oxidation by chromic acid. The second pathway is postulated for our *Streptomyces* type but the chemical oxidation of this type is not yet known. However, it is well known that in Oppenauer oxidation of bile acid the first step of the oxidation of the secondary hydroxyl groups in cholic acid (*17*) or desoxycholic acid (*18*, *19*) is not at C_7 or C_{12} but at C_3, and also that the oxidation product of methyl cholate by potassium permanganate is not methyl 3α-hydroxy-7,12-diketocholanate but methyl 12α-hydroxy-3,7-diketocholanate (*20*). Such evidences suggest the presence of the second oxidative pathway of cholic acid by microorganisms.

In our experiments, the (axial) 7α-hydroxyl group in cholic acid is not attacked even after the β-oxidation of a side chain of it. This finding indicates that the first oxidation product of cholic acid by *S. gelaticus* 1164 may be either 3α,7α-dihydroxy-12-ketocholanic acid or 7α,12α-dihydroxy-3-ketocholanic acid. Also it is known that the 3α-hydroxyl group in cholic acid is an equatorial bond and the 12α-hydroxyl group is an axial bond. Furthermore, Magasanik *et al.* (*21*) has discovered a most interesting correlation between the conformations of cyclitols and their liability to oxidation by *Acetobacter suboxidans* and stated that only axial hydroxyl groups were oxidized. On the basis of these evidences, it is tentatively suggested that the first step of the oxidation of cholic acid in the second pathway is 3α,7α-dihydroxy-12-ketocholanic acid. While, as well as Oppenauer oxidation (*17–19*) and permanganate oxidation (*20*) it may be suggested that 7α,12α-dihydroxy-3-ketocholanic acid is the first mirobial oxidation product from cholic acid in the second pathway. In both cases, the second oxidation product is probably 7α-hydroxy-3,12-diketocholanic acid and its 7α-hydroxyl group seems to be intact until β-oxidation of the side chain of this acid occurs.

Therefore, such observations suggest that an intermediate containing 7α-hydroxyl group or 7α-hydroxy-3-keto-Δ^4-ene grouping plays

an important role for the degradation of cholic acid by our seven *Streptomyces spp*. In order to confirm this pathway by *Streptomyces*, the isolation of the intermediate derivatives formed during the oxidation of cholic acid to 7α-hydroxy-3,12-diketo-Δ^4-bisnorcholenic acid is under way. The expected intermediates, namely, 7α,12α-dihydroxy-3-ketocholanic acid, 3α,7α-dihydroxy-12-ketocholanic acid, 7α-hydroxy-3,12-diketocholanic acid and some unidentified substances have been isolated from the culture filtrate obtained after incubation of cholic acid with *S. gelaticus* 1164. These data will be reported in elsewhere (*22*).

SUMMARY

1. The culture conditions for the formation of a new unsaturated C-22 acid (presumably 7α-hydroxy-3,12-diketo-Δ^4-bisnorcholenic acid) from cholic acid by *S. gelaticus* 1164 were investigated by shake bottle culture.

2. All of the seven *Streptomyces spp*. which were identified in the previous paper (*6*) degraded cholic acid through an intermediate containing 7α-hydroxy-3-keto-Δ^4-ene grouping. This fact was confirmed through the spectrophotometric analyses of the cultures.

3. Ability of the above seven *Streptomyces spp*. to utilize the various kinds of bile acids was investigated and it was found that the bile acids containing three functional groups at C_3, C_7 and C_{12} were utilized except norcholic and homocholic acids. This fact suggests that the utilization of bile acids by *Streptomyces* is dependent on both the nucleus constitution and the length of side chains of bile acid.

4. An oxidative pathway of cholic acid by *Streptomyces* was discussed.

The authors express their hearty thanks to Prof. T. Shimizu and Prof. S. Mizuhara for their kind guidances.

REFERENCES

(*1*) Hayakawa, S., *Proc. Japan Acad.*, **30**, 128 (1954)
(*2*) Hayakawa, S., *Proc. Japan Acad.*, **30**, 133 (1954)
(*3*) Hayakawa, S., *Proc. Japan Acad.*, **30**, 139 (1954)
(*4*) Halperin, A. H., Quastel, J. H., and Scholefield, P. G., *Arch. Biochem. Bioph.*, **52**, 5 (1954)
(*5*) Hayakawa, S., Saburi, Y., Fujii, T., and Sonoda, Y., *J. Biochem.*, **43**, 723 (1956)
(*6*) Saburi, Y., Hayakawa, S., and Fujii, T., *Japan J. Exp. Med.*, **25**, 151 (1955)

(7) Peterson, D. H., Microorganisms and steroid transformations in perspectives and horizons in microbiology, Rutgers Univ. Press, p. 121 (1955)
(8) Gallagher, T. F., and Long, W. P., *J. Biol. Chem.*, **147**, 131 (1943)
(9) Kaziro, K., and Shimada, T., *Z. Physiol. Chem.*, **249**, 220 (1937)
(10) Hoehn, W. M., and Schneider, A. W., U. S. Patent 2,231,598 (1943)
(11) Haslewood, G.A.D., *Nature*, **150**, 211 (1942)
(12) Haslewood, G.A.D., *Biochem. J.* **37**, 109 (1943)
(13) Schmidt, L. H., Hughes, H. B., Green, M. H., and Cooper, E., *J. Biol. Chem.*, **145**, 229 (1942)
(14) Schmidt, L. H., and Hughes, H. B., U. S. Patent, 2,360,447 (1944)
(15) Hoehn, W. M., Schmidt, L. H., and Hughes, H. B., *J. Biol. Chem.*, **152**, 59 (1944)
(16) Hayakawa, S., and Morimoto, S., *Collected Papers Hiroshima Med. Sch.*, **2**, 9 (1950)
(17) Kuwada, S., and Morimoto, S., *Bull. Chem. Soc. Japan*, **17**, 147 (1942)
(18) Fuchs, H. G., and Reichstein, T., *Helv. Chem. Acta*, **26**, 511 (1943)
(19) Wakabayashi, G., *J. Japan Biochem. Soc.*, **22**, 211 (1950)
(20) Kuwada, S., Furuki, S., and Kawashima, M., *Ann. Reports Takeda Research Lab.*, **8**, 50 (1949)
(21) Magasanik, B., and Chargaff, E., *J. Biol. Chem.*, **174**, 173 (1948)
(22) Hayakawa, S., Saburi, Y., and Teraoka, H., *Proc. Japan Acad.*, **32**, 519 (1956)

The Journal of Biochemistry, Vol. 43, No. 5, 1956

MICROBIOLOGICAL DEGRADATION OF BILE ACIDS

VI. ON THE STRUCTURE OF THE UNSATURATED C-22 ACID TRANSFORMED FROM CHOLIC ACID BY S. GELATICUS 1164

By SHOHEI HAYAKAWA*, YASUO SABURI**, TAKAO FUJII* and YOSHIYA SONODA*

(*Department of Biochemistry, Okayama University Medical School, Okayama, and **First Bacteriological Division, The Institute for Infectious Disease, The University of Tokyo, Tokyo)

(Received for publication, July 5, 1956)

In the previous papers (*1–3*), it was demonstrated that a new unsaturated C-22 acid of m.p. 280–282° (decomp.) was obtained from cholic acid by the action of *S. gelaticus* 1164. The new compound was presumed to be 7α-hydroxy-3,12-diketo-$\Delta^{4,9(11)}$-bisnorcholadienic acid, but there was no definite proof on the positions of the two double bonds in the cholane nucleus. Recently the ultraviolet and infrared absorption characteristics of the new compound and a degradation product of it were investigated, and the results obtained showed that the new acid may be 7α-hydroxy-3,12-diketo-Δ^{4}-bisnorcholenic acid.

EXPERIMENTAL

Acid with M. P. 280–282° (Decomp.) and Its Methyl Ester

Sodium cholate (0.3 g./dl.) was incubated with *S. gelaticus* 1164 by a shaking culture as described in the previous paper (*4*), and the new acid of m. p. 280–282° (decomp.) was isolated by the same method as described in the first paper (*1*) of this series. The infrared spectrum (Fig. 1-A) of this acid showed the absorption bands (in Nujol) at the following wavelengths: 3.00, 5.83, 6.05, 6.21 and 6.25 μ. As this acid was insoluble in chloroform, the measurement of the infrared spectrum in chloroform was impossible. The infrared spectrum (Fig. 1-B) of the methyl ester, m.p. 244–246° (decomp.), of this acid (*1*) showed the following absorption bands (in Nujol): 2.99, 5.75, 5.87, 5.99, 6.175 and 6.24 μ.

Dehydration Product of the Above Acid and Its Methyl Ester

Formation of Acid by Caustic Alkali—Four hundred and eighty milli-

grams of the methyl ester, m. p. 244–246° (decomp.), was dissolved in 100 ml. of warm methanol. To this solution 4.5 g. of potassium hydroxide dissolved in 50 ml. of water was added and the mixture was refluxed on a boiling water bath for 6 hours. The resulting deep red-brown solution was cooled, neutralized with dilute hydrochloric acid, concentrated in vacuum to about 40 ml. and acidified by dropwise addition of dilute hydrochloric acid. After cooling, the crystalline precipitate was collected and washed. Crystallization from ethyl acetate gave 150 mg. of yellow needles, m. p. 240–250° (decomp.). Twice recrystallizations from acetone gave pale yellow needles sintering at 240° and melting at 250–252° with decomposition, $\lambda_{max.}^{alc.}$ 280.5 mμ (log ε 4.43). The infrared spectrum (Fig. 1-C) of this acid for carboxyl and ketone groups showed the following two bands (in Nujol): 5.805 and 5.88 μ, and there was no hydroxyl band. Furthermore, the spectrum (in Nujol) for $\Delta^{4,6}$-3-ketone group in this acid showed only two bands (6.11 and 6.19 μ) and an absorption at around 6.3 μ was not clear, but showed characteristic $\Delta^{4,6}$-3-ketone absorption at the fingerprint region.

Analysis.

Calcd. for $C_{22}H_{28}O_4$: C, 74.13; H, 7.92.
Found: C, 74.03; H, 8.13.

The dienone acid above obtained was also obtained by refluxing the above original acid, m. p. 280–282° (decomp.), with aqueous caustic alkali. The saponification and dehydration of the methyl ester, m. p. 244–246° (decomp.), by alcoholic potassium carbonate was incomplete in one hour and gave a mixture of this dienone acid, m. p. 250–252° (decomp.), and the unsaponified original ester.

The methyl ester of this dienone acid, prepared by esterification with diazomethane in ether by the usual method, was crystallized from methanol in pale yellow, prismatic needles, m. p. 182–184° without decomposition, $\lambda_{max.}^{alc.}$ 280.5 mμ (log ε 4.45). The infrared spectrum showed the following absorption bands (in Nujol): ester and ketone, 5.79 and 5.84 μ; $\Delta^{4,6}$-3-ketone; 6.04, 6.18 and 6.33 μ. This spectrum (Fig. 1-D) showed no hydroxyl absorption, but characteristic $\Delta^{4,6}$-3-ketone absorption both at around 6 μ and at the fingerprint region.

Analysis.

Clacd. for $C_{23}H_{30}O_4$: C, 74.56; H, 8.16.
Found: C, 74.49; H, 8.30.

Formation of Ester by Sulfuric Acid—A suspension of 200 mg. of the C-22 acid, m. p. 280–282° (decomp.), in 4 ml. of methanol containing

two drops of concentrated sulfuric acid was refluxed on a boiling water bath for 30 minutes. Soon the acid dissolved into the reaction mixture. The resulting green solution was diluted with dilute sodium bicarbonate solution and the crystalline precipitate which was separated on standing, was collected, washed with water and dried. The filtrate and washings were combined and stored for the later experiment. Crystallization of the dried precipitate from ethyl acetate afforded 70 mg. of pale yellow needles of m. p. 179–181°. This methyl ester gave no depression of melting point with the methyl ester, m. p. 182–184°, of the dienone acid, m. p. 250–252° (decomp.), obtained from the C-22 acid by refluxing with methanolic potassium hydroxide and further an identification of the both esters was confirmed by infrared spectrum.

Analysis.

Calcd. for $C_{23}H_{30}O_4$: C, 74.56; H, 8.16.
Found: C, 74.46; H, 8.53.

The combined filtrates were acidified with dilute hydrochloric acid and kept at O° for several hours. The resulting precipitate was collected, washed with a small volume of water and dried. Crystallization of the dried substance from acetone gave 12 mg. of pale yellow needles sintering at 240° and melting at 249–251° with decomposition. This acid gave no depression of melting point with the acid, m. p. 250–252° (decomp.), obtained from the C-22 acid with potassium hydroxide and infrared analysis confirmed the identity of these acids.

Analysis.

Calcd. for $C_{22}H_{28}O_4$: C, 74.13; H, 7.92.
Found: C, 74.44; H, 8.18.

For this dehydration reaction which was catalyzed with proton, the following procedure was adopted and the same result as described above was obtained. A suspension of 100 mg. of methyl ester, m. p. 244–246° (decomp.), of the C-22 acid in 5 ml. of methanol containing three drops of concentrated sulfuric acid was left to stand at room temperature. The ester dissolved into the reaction mixture within 24 hours. After four days, the solution was diluted with dilute sodium bicarbonate solution and the crystalline precipitate was collected, washed with water and dried. By acidifying the filtrate and washings no precipitate occurred. Crystallization of the dried substance afforded 80 mg. of colorless needles of m. p. 181–183°. This ester gave no depression of melting point with the ester obtained from the C-22 acid by sulfuric acid or potassium hydroxide and infrared analysis confirmed

the identity of the three esters.

Analysis.

Calcd. for $C_{23}H_{30}O_4$: C, 74.56; H, 8.16.
Found: C, 74.46; H, 8.37.

Infrared Spectra

The infrared spectra of this experiment were measured kindly by Mr. Y. Matsui of Research Laboratory, Shionogi & Co., Ltd. with a Perkin-Elmer Spectrophotometer, model 12 C.

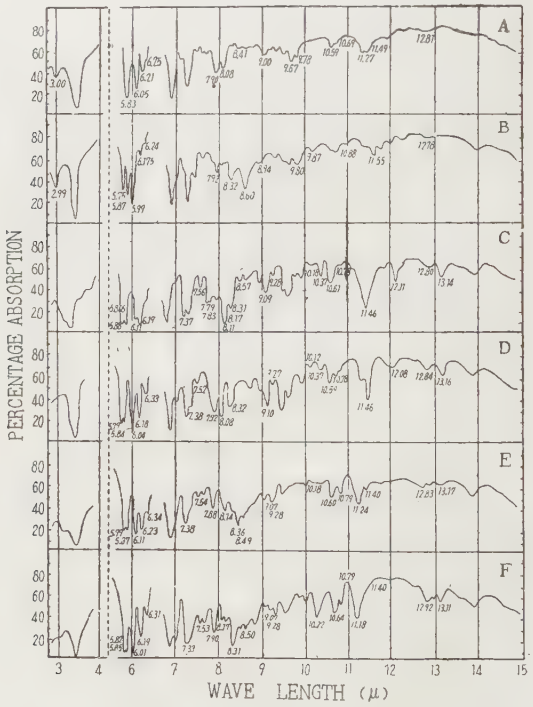


FIG. 1. Infrared spectra in Nujol mull: A; C-22 acid, B; methyl ester of the C-22 acid, C; dehydration product (acid) of the C-22 acid, D; dehydration product (methyl ester) of the C-22 acid, E; 3,12-diketo-$\Delta^{4,6}$-choladienic acid (synthetic sample), F; methyl 3,12-diketo-$\Delta^{4,6}$-choladienate (synthetic sample).

DISCUSSION

Ultraviolet absorption analysis played an important role in substantiating the structures of steroids. Dorfman (*5*) in a noteworthy paper on the ultraviolet absorption spectra of steroids states that a Δ^4-3-ketone generally absorbs selectively at 241 mμ with an average molecular extinction coefficient of 16,600 (*e.g.*, methyl 12α-hydroxy-3-keto-Δ^4-bisnorcholenate, $\lambda_{max.}^{alc.}$ 241 mμ, ε 14,000), whereas a $\Delta^{9(11)}$-12-ketone absorbs selectively at 240 mμ with an average molecular extinction coefficient of 11,200 (*e. g.*, methyl 3α-hydroxy-12-keto-$\Delta^{9(11)}$-cholenate, $\lambda_{max.}^{alc.}$ 240 mμ, ε 12,000). Further, it is well known that $\Delta^{4,16}$-pregnadiene-3,20-dione (*6*) which contains two isolated α,β-unsaturated ketonic chromophores, namely, Δ^4-3-ketone and Δ^{16}-20-ketone absorbs selectively at 241 mμ, with a molecular extinction coefficient of 25,200.

As described in the previous papers (*1–3*), the new unsaturated C-22 acid, m. p. 280–282° (decomp.), was presumed to be 7α-hydroxy-3,12-diketo-$\Delta^{4,9(11)}$-bisnorcholadienic acid and the ultraviolet absorption spectrum of the acid showed a maximum at 240.3 mμ (ε 11,500) in alcohol. The presence of a bile acid containing two chromophores of Δ^4-3-ketone and $\Delta^{9(11)}$-12-ketone is not yet known, but the molecular extinction coefficient of the C-22 acid is about half compared with that of $\Delta^{4,16}$-pregnadiene-3,20-dione which has two chromophores in the molecule. Thus the $\Delta^{4,9(11)}$-3,12-diketone structure postulated for the C-22 acid is now doubtful. So the infrared absorption spectra of this acid and its methyl ester were determined in order to confirm the presence of two chrompophores of Δ^3-3-ketone and $\Delta^{9,(11)}$-12-ketone in these molecules. The spectrum (Fig. 1-A) of the acid showed the following absorption bands: hydroxyl, 3.00 μ; carboxyl and six-membered cyclic ketone, 5.83 μ; conjugated ketone, 6.05 μ; conjugated double bond, 6.21 and 6.25 μ, and the spectrum (Fig. 1-B) of the methyl ester showed the following absorption bands which is similar to the original acid: hydroxyl, 2.99 μ; ester and ketone, 5.75 and 5.87 μ; conjugated ketone, 5.99 μ; conjugated double bond, 6.175 and 6.24 μ. The band systems centered around 6 μ of both acid and methyl ester indicated the presence of a non-conjugated ketone in those molecules and displayed bands at approximately 6.00, 6.17 and 6.24 μ, this triad being characteristic of the $\Delta^{1,4}$-3-ketone grouping (*7, 8*). So the possibility of the structure of $\Delta^{4,9(11)}$-3,12-diketone postulated previously for the C-22 acid was eliminated.

From a consideration of the above-stated absorption characteristic it seems most reasonable to conclude that the C-22 acid is 7α-hydroxy-3,12-diketo-$\Delta^{1,4}$-bisnorcholadienic acid, because it was confirmed synthetically (*3*) that the hydrogenated C-22 acid was 7α-hydroxy-3,12-diketobisnorcholanic acid. Recently, Jones *et al.* (*9*) reported the infrared spectra of keto-steroids at the region of longer wave length (7–15 μ). The band systems at the fingerprint region of the C-22 acid and its methyl ester as shown in Fig. 1-A and 1-B does not coincide with the band systems of various $\Delta^{1,4}$-3-keto-steroids examined by Jones *et al.* (*9*), but that resemble with the spectrum of Δ^{4}-3-ketone rather than that of $\Delta^{1,4}$-3-ketone. Further, since a partial synthesis of 7α-hydroxy-3,12-diketo-$\Delta^{1,4}$-bisnorcholadienic acid from cholic acid is rather cumbersome, it was thought advisable to investigate a degradation product of this C-22 acid for establishing a constitution of the C-22 acid.

Fieser *et al.* (*10*) reported that methyl 3α,7α-diacetoxy-12-keto-$\Delta^{9(11)}$-cholenate affords 3α-hydroxy-12-keto-$\Delta^{7,9(11)}$-choladienic acid by heating with caustic alkali and stated that the facile dehydration reaction of C_7α-hydroxyl (or acetoxyl) with C_8-hydrogen atom is attributable to activation of the C_8-hydrogen atom by 9,11-double bond. Also Greenhalgh *et al.* (*11*) reported that the only product isolated from the hydrolysis of 7α-hydropyranyloxy-Δ^{4}-cholestene-3-one was $\Delta^{4,6}$-cholestadiene-3-one, the 7α-hydroxy-Δ^{4}-cholestene-3-one, presumably formed initially, having undergone dehydration in the acidic medium. These dehydration reactions were applied for the elimination of the C_7α-hydroxyl groups of the C-22 acid and its methyl ester, and the dehydration products were examined. The dehydration product of the C-22 acid and its methyl ester by caustic alkali was obtained in crystalline form, and the data of the microanalysis gave a formula $C_{22}H_{28}O_4$, its ultraviolet absorption spectrum showed the maximum at 280.5 mμ (log ε 4.43), as exhibited by $\Delta^{4,6}$-3-ketones and the infrared spectrum (Fig. 1-C) showed characteristic $\Delta^{4,6}$-3-ketone absorption although an absorption at around 6.3 μ was not clear. Furthermore, the methyl ester of the above-described dehydration product by alkali was identical with the methyl esters obtained from the C-22 acid and its methyl ester through the dehydration reaction by sulfuric acid and the both esters coincided with a formula $C_{23}H_{30}O_4$, its ultraviolet absorption spectrum showed a maximum at 280.5 mμ (log ε 4.45) and the infrared spectrum showed the following characteristic $\Delta^{4,6}$-3-ketone absorption both at around 6 μ and at fingerprint region as described in Fig. 1-D : ester and C_{12}-ketone, 5.79 and 5.84 μ ; $\Delta^{4,6}$-3-ketone, 6.04, 6.18 and 6.33 μ.

From these microanalysis data and ultraviolet and infrared absorption characteristics (*5*, *8*, *12*), it was suggested that the 7α-hydroxyl group in the C-22 acid was easily dehydrated to occur 3,12-diketo-$\Delta^{4,6}$-bisnorcholadienic acid by catalytic action of hydroxyl ion or proton. In order to confirm the presence of $\Delta^{4,6}$-3,12-diketone group in the dehydration product, 3,12-diketo-$\Delta^{4,6}$-choladienic acid and its methyl ester were prepared from cholic acid (*13*), and the infrared absorption spectra (Fig. 1-E and 1-F) of the compounds were determined. The comparison with the absorption spectra of the dehydration products of the C-22 acid and its methyl ester and that of the authentic 3,12-diketo-$\Delta^{4,6}$-choladienic acid and its methyl ester indicated that $\Delta^{4,6}$-3-ketone group is present in the dehydration product. So the posibility of the structure of $\Delta^{1,4}$-3,12-diketone postulated above for the C-22 acid was eliminated.

In view of the above-stated facts the most reasonable conclusion to be drawn from available data is that the new unsaturated C-22 acid is 7α-hydroxy-3,12-diketo-Δ^{4}-bisnorcholenic acid and 7α-hydroxyl group in the acid is probably dehydrated to produce 3,12-diketo-$\Delta^{4,6}$-bisnorcholadienic acid by the action of caustic alkali or sulfuric acid as shown in the following Fig. 2.

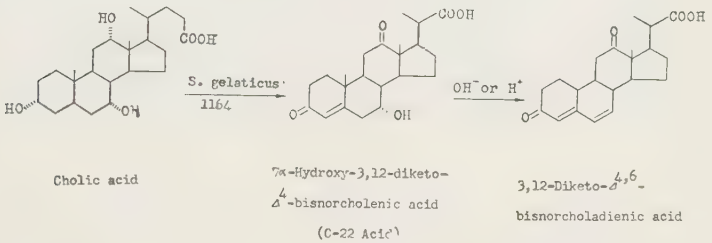


FIG. 2. Formation of the C-22 acid from cholic acid by *S. gelaticus* 1164 and dehydration of the C-22 acid by caustic alkali or sulfuric acid.

SUMMARY

S. gelaticus 1164 converts cholic acid to a new unsaturated C-22 acid with m. p. 280–282° (decomp.) which was previously postulated as 7α-hydroxy-3,12-diketo-$\Delta^{4,9(11)}$-bisnorcholadienic acid. Further investigations demonstrated that the new acid may be 7α-hydroxy-3,12-diketo-Δ^{4}-bisnorcholenic acid.

The authors express their hearty thanks to Prof. T. Shimizu and Prof. S. Mizuhara for their kind guidances, and to Mr. Y. Matsui of Research Laboratory, Shionogi & Co., Ltd. for his help in measuring the infrared absorption spectra and for his valuable criticism.

REFERENCES

(1) Hayakawa, S., *Proc. Japan Acad.*, **30**, 128 (1954)
(2) Hayakawa, S., *Proc. Japan Acad.*, **30**, 133 (1954)
(3) Hayakawa, S., *Proc. Japan Acad.*, **30**, 139 (1954)
(4) Saburi, Y., Hayakawa, S., Fujii, T., and Akaeda, I., *J. Biochem.*, **43**, 711 (1956)
(5) Dorfman, L., *Chem. Rev.*, **53**, 47 (1953)
(6) Allen, W. S., and Bernstein, S., *J. Am. Chem. Soc.*, **77**, 1028 (1955)
(7) Fried, J., Thoma, R. W., and Klingsberg, A., *J. Am. Chem. Soc.*, **75**, 5764 (1953)
(8) Jones, R. N., Humpheries, P., Fackard, E., and Dobriner, K., *J. Am. Chem. Soc.*, **72**, 86 (1950)
(9) Jones, R. N., Herling, F., and Katzenellenbogen, E., *J. Am. Chem. Soc.*, **77**, 651 (1955)
(10) Fieser, L. F., Rajagopalan, S., Wilson, E., and Tishler, M., *J. Am. Chem. Soc.*, **73**, 4133 (1951)
(11) Greenhalgh, C. W., Henbest, H. B., and Jones, E.R.H., *J. Chem. Soc.*, 2375, (1952)
(12) Jones, R. N., and Herling, F., *J. Org. Chem.*, **19**, 1252 (1954)
(13) Hayakawa, S., Saburi, Y., Fujii, T., and Sonoda, Y., *J. Biochem.*, **43**, 731 (1956)

The Journal of Biochemistry, Vol. 43, No. 5, 1956

MICROBIOLOGICAL DEGRADATION OF BILE ACIDS

VII. PARTIAL SYNTHESIS OF 3,12-DIKETO-$\Delta^{4,6}$-CHOLADIENIC ACID AND ITS DERIVATIVES FROM CHOLIC ACID

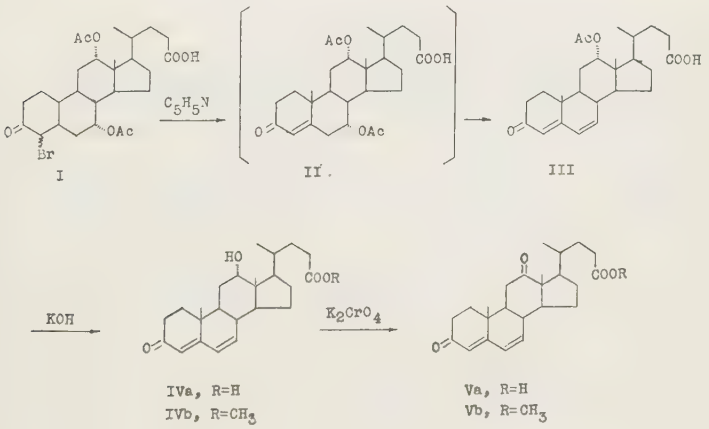
By SHOHEI HAYAKAWA*, YASUO SABURI**, TAKAO FUJII* and YOSHIYA SONODA*

(*Department of Biochemistry, Okayama University Medical School, Okayama, and **First Bacteriological Division, The Institute for Infectious Disease, The University of Tokyo, Tokyo)

(Received for publication, July 5, 1956)

In a previous paper (*1*), it was pointed out that the acid with m.p. 280–282° (decomp.) transformed from cholic acid by the action of *S. gelaticus* 1164 appears to produce 3,12-diketo-$\Delta^{4,6}$-bisnorcholadienic acid, $C_{22}H_{28}O_4$, by treating with caustic alkali or mineral acid It is thought advisable to synthesize a bile acid having a $\Delta^{4,6}$-3,12-diketone group for a comparison of the newly obtained bisnor-dienone acid with a known $\Delta^{4,6}$-3,12-diketo-bile acid, so the present report is designed to yield information on the partial synthesis of 3,12-diketo-$\Delta^{4,6}$-choladienic acid, $C_{24}H_{32}O_4$, and its derivatives from cholic acid as described in the following chart.

The newly prepared 3,12-diketo-$\Delta^{4,6}$-choladienic acid and its methyl ester were applied to assume that the new acid with m. p. 280–



282° (decomp.) obtained from cholic acid through microbiological degradation is 7α-hydroxy-3,12-diketo-Δ^4-bisnorcholenic acid as described in a previous paper (*1*).

EXPERIMENTAL

3-Keto-4 ξ-bromo-7α,12α-diacetoxycholanic Acid (***I***)—This compound of m. p. 207–209° (decomp.) was prepared from cholic acid by a procedure similar to that described by Matsumoto (*2*) and used for the next step without further purification.

3-Keto-12α-acetoxy-$\Delta^{4,6}$-choladienic Acid (***III***)—The following procedure modified from the method of Matsumoto (*2*) was adopted: Ten g. of bromo-compound **I** was dissolved in 100 ml. of dry pryidine and refluxed for 15 hours on a sand bath. The orange reaction mixture was concentrated in vacuum to about one-half of the initial volume and poured into about 500 ml. of water. The resulting cloudy solution was acidified with dilute hydrochloric acid and extracted with an acetic ester—alcohol mixture (5:1). The acetic ester layer was separated, washed with water, dried over anhydrous sodium sulfate and concentrated, giving pale yellow needles which was filtered and washed with acetic ester, yield 1.6 g., m. p. 210–212°. Two recrystallizations from methanol gave a laminar and almost colorless crystal melting at 211–213°, $\lambda_{max.}^{alc.}$ 283 mμ (log ε 4.42). The infrared spectrum showed the following absorption bands (in Nujol): acetate ester and carboxyl, 5.755 and 5.79 μ; $\Delta^{4,6}$-3-ketone, 6.11, 6.21 and 6.30 μ.

Analysis.

Calcd. for $C_{26}H_{36}O_5$: C, 72.86; H, 8.47.
Found: C, 73.35; H, 8.65.

This compound was described as 3-keto-12α-acetoxy-$\Delta^{4,7}$-choladienic acid of m. p. 212° by Matsumoto (*2*).

The methyl ester of **III** was prepared with diazomethane and crystallized from acetic ester—petroleum ether, in almost colorless prisms, m. p. 150–151.5°, $\lambda_{max.}^{alc.}$ 283 mμ (log ε 4.42). The infrared spectrum of this ester showed the following absorption bands (in Nujol): acetate ester and methyl ester, 5.76 and 5.79 μ; $\Delta^{4,6}$-3-ketone, 6.04, 6.185 and 6.31 μ.

Analysis.

Calcd. for $C_{27}H_{38}O_5$: C, 73.27; H, 8.65.
Found: C, 73.35; H, 8.79.

3-Keto-12α-hydroxy-$\Delta^{4,6}$-choladienic Acid (***IVa***)—A mixture of 2 g. of the dienone acetate (**III**) and 2 g. of potassium hydroxide in 40 ml. of water was refluxed for two hours on a sand bath and the yellow solution was cooled and acidified by dropwise addition of dilute hydrocholric acid. After cooling, semicrystalline dienone acid was collected and washed with water. After drying, crystallization from alcohol gave 1.5 g. of pale yellow, prismatic needles, m. p. 252–254° and $\lambda_{max.}^{alc.}$ 284.5 mμ (log ε 4.42).

Analysis.

Calcd. for $C_{24}H_{34}O_4$: C, 74.57; H, 8.87.
Found: C, 74.66; H, 9.02.

The methyl ester **IVb** of **IVa** was prepared with diazomethane and crystallized from methanol, in pale yellow, prismatic needles, m. p. 184–185.5° and $\lambda_{max.}^{alc.}$ 284 mμ (log ε 4.42). The infrared spectrum showed the following absorption bands (in Nujol): hydroxyl, 2.845 μ; methyl ester, 5.82 μ; $\Delta^{4,6}$-3-ketone, 6.01, 6.19 and 6.34 μ.

Analysis.

Calcd. for $C_{25}H_{36}O_4$: C. 74.96; H, 9.06.
Found: C, 74.52; H, 8.73.

Saponification with potassium carbonate in aqueous alcohol of this ester afforded the parent dienone acid with m .p. 253–255° and $\lambda_{max.}^{alc.}$ 284.5 mμ (log ε 4.42) without any rearrangement.

Methyl 3,12-Diketo-$\Delta^{4,6}$-choladienate (***Vb***)—A solution of 320 mg. of potassium chromate in 1 ml. of water was added to solution of 500 mg. of the dienone ester **IVb** in 20 ml. of glacial acetic acid and the solution was let stand at room temperature (about 27°) for 14 hours and then diluted with 70 ml. of water. The crystalline precipitate on crystallization from alcohol afforded 350 mg. of light yellow prisms, m. p. 173–175°. Repeated crystallization from methanol gave almost colorless prismatic needles, m. p. 174–175°, $\lambda_{max.}^{alc.}$ 280.5 mμ (log ε 4.37). The infrared spectrum showed absorption bands (in Nujol) as follows: methyl ester and C_{12}-ketone, 5.82 and 5.85 μ; $\Delta^{4,6}$-3-ketone, 6.01, 6.19 and 6.31 μ.

3,12-Diketo-$\Delta^{4,6}$-choladienic Acid (***Va***) *from* ***IVa***—To a solution of 200 mg. of the dienone acid **IVa** in 18 ml. of glacial acetic acid was added a solution of 130 mg. of potassium chromate in 0.5 ml. of water and the mixture was let stand at room temperature (about 27°) for 20 hours and diluted with water. The crystalline precipitate on crystalli-

zation from acetic ester afforded 140 mg. of colorless product sintering at 200° and melting at 206–208°. Two crystallizations from alcohol —petroleum ether gave colorless prisms, m. p. 206–208°, $\lambda_{max.}^{alc.}$ 281 mμ (log ε 4.40). The infrared spectrum showed absorption bands (in Nujol) as follows; carboxyl and C_{12}-ketone, 5.79 and 5.87 μ; $\Delta^{4,6}$-ketone, 6.11, 6.23 and 6.34 μ.

Analysis.

Calcd. for $C_{24}H_{32}O_4$: C, 74.97; H, 8.39.
Found: C, 75.13; H, 8.49.

The methyl ester **Vb** was obtained by the reaction with diazomethane on a solution of the above acid **Va** in methanol and crystallized from methanol; m. p. 175–177°, $\lambda_{max.}^{alc.}$ 280.5 mμ (log ε 4.37) without depression on admixture with **Vb** derived from **IVb**.

Analysis.

Calcd. for $C_{25}H_{34}O_4$: C, 75.34; H, 8.60.
Found: C, 75.14; H, 8.69.

3,12-*Diketo-*$\Delta^{4,6}$*-choladienic Acid* (***Va***) *from* ***Vb***—Two hundred mg. of methyl 3,12-diketo-$\Delta^{4,6}$-choladienate **Vb** obtained from **IVb** was refluxed for two hours with 300 mg. of potassium carbonate in 30 ml. of alcohol and 15 ml. of water. The resulting yellow solution was concentrated in vacuum to about 15 ml., diluted with 15 ml. of water and acidified with dilute hydrochloric acid. The resultant precipitate was filtered, washed and dried. Crystallization from alcohol—petroleum ether gave 100 mg. of acid, m. p. 206–208°, $\lambda_{alc.}^{max.}$ 281 mμ (log ε 4,40). A mixed m. p. with **Va** derived from **IVa** showed no depression.

Analysis.

Calcd. for $C_{24}H_{32}O_4$: C, 74.97; H, 8.39.
Found: C, 75.03; H, 8.71.

DISCUSSION

Matsumoto (*2*) reported that 3-keto-12α-acetoxy-$\Delta^{4,7}$-choladienic acid was obtained from 3-keto-4ξ-bromo-7α,12α-diacetoxycholanic acid (**I**) by dehydrobromination in boiling pyridine. In the course of this reaction, the authors found that the expected dehydrobromination product, 3-keto-7α,12α-diacetoxy-Δ^4-cholenic acid (**II**), was not obtained but an acid containing two double bonds was obtained and the maximum of the ultraviolet absorption spectrum of the dehydrobromi-

nation product of **I** was 283 mμ (log ε 4.42). From the consideration of the data on the ultraviolet spectrum (*3, 4*) and the microcombution data it seems most reasonable to conclude that **I** is dehydrobrominated into an intermediate (**II**) by pyridine, the newly introduced 4,5-double bond activates the C_6-hydrogen atoms and elimination of the elements of acetic acid from the (axial) $C_6\beta$-hydrogen atom and the (axial) $C_7\alpha$-acetoxyl group in **II** proceeds to form a dienone acid **III**. So the newly introduced two double bonds must be at the 4,5- and 6,7-positions, as in **III**. This structure is consistent with the presence in the spectrum of an intense absorption band at 283 mμ, since the maximum calculated (*3, 4*) for **III** from available analogs is 280 mμ. As reported by Matsumoto (*2*), if it was presumed that the dienone acid **III** will be 3-keto-12α-acetoxy-$\Delta^{4,7}$-choladienic acid, which must show a maximum at about 237–238 mμ in the absorption spectrum (*4, 5*). The dienone acid **III** and its methyl ester exhibited infrared absorption spectra at 6.11, 6.21 and 6.30 μ (in Nujol) and 6.04, 6.185 and 6.31 μ (in Nujol), respectively. This observation conforms with the generalization that $\Delta^{4,6}$-3-keto-steroids exhibit the absorption maxima at 5.99–6.00, 6.175–6.19 and 6.30 μ (in $CHCl_3$) (*6*). These data on the ultraviolet and infrared absorption spectra show that the dehydrobromination product **III** of **I** with pyridine is 3-keto-12α-acetoxy-$\Delta^{4,6}$-choladienic acid and that the possibility of the formation of Matsumoto's acid (*2*), 3-keto-12α-acetoxy-$\Delta^{4,7}$-choladienic acid, from **I** is eliminated completely.

The compounds, **IVa**, **IVb**, **Va** and **Vb** were prepared from **III** by the usual manner. As described in the experimental part, the ultraviolet and infrared absorption characterstics found for them indicated that in the course of **Vb** from **III**, any rearrangement of the two double bonds in **III** was not occurred, supporting the assigned structures.

The present investigation, further, appeared of interest for the following experimental evidence. During the continuation of our survery of the intermediate metabolism of bile acids by *Streptomyces*, a new intermediate product of cholic acid was separated recently as methyl ester. The new ester was identical with methyl 3,12-diketo-$\Delta^{4,6}$-choladienate prepared here (*7*).

SUMMARY

1. The identification of the dehydrobromination product of 3-keto-4ξ-bromo-7α,12α-diacetoxycholanic acid with pyridine was attempted and it was shown that the product having the dienone group-

ing is 3-keto-12α-acetoxy-$\Delta^{4,6}$-choladienic acid and it is not in the structure of $\Delta^{4,7}$-3-ketone reported by Matsumoto (*2*).

2. The partial syntheses of 3,12-diketo-$\Delta^{4,6}$-choladienic acid and its derivatives from cholic acid were described, and these compounds were used to confirm the structure of the various intermediates of cholic acid obtained through the action of *S. gelaticus* 1164.

The authors express their hearty thanks to Prof. T. Shimizu and Prof. S. Mizuhara for their interests on this study and their encouragements, and to Mr. Y. Matsui of Research Laboratory, Shionogi & Co., Ltd. for his help in measuring the infrared absorption spectra and for his valuable criticism.

REFERENCES

(*1*) Hayakawa, S., Saburi, Y., Fujii, T., and Sonoda, Y., *J. Biochem.*, **43**, 723 (1956)
(*2*) Matsumoto, K., *J. Biochem.*, **36**, 183 (1944)
(*3*) Fieser, L. F., and Fieser, M., Natural products related to phenanthrene, Reinhold Pub. Co., 2nd ed., p. 192 (1949)
(*4*) Dorfman, L., *Chem. Rev.*, **53**, 47 (1953)
(*5*) Bernstein, S., Littell, R., and Williams, J. H., *J. Org. Chem.*, **18**, 1418 (1953)
(*6*) Jones, R. N., and Herling, F., *J. Org. Chem.*, **19**, 1252 (1954)
(*7*) Hayakawa, S., Saburi, Y., and Teraoka, H., *Proc. Japan Acad.*, **32**, 519 (1956)

The Journal of Biochemistry, Vol. 43, No. 5, 1956

HALF CYSTINE, AN ADDITIONAL *N*-TERMINAL GROUP OF SERUM ALBUMIN

Sirs:

The application of the usual dinitrophenyl (DNP) or phenylthiocarbamyl (PTC) method on serum albumin was reported to have given only one aspartic acid residue as the *N*-terminal group (*1*, *2*, *3*), and the *N*- and *C*-terminal sequences were examined on the assumption that the protein consists of one peptide chain (*3*, *4*). Some evidences, however, have now been obtained that one amino group of the cystine residues is free, together with one of the aspartic acid residues in equine serum albumin. The ether extract of the acid hydrolysate of DNP-serum albumin (12 *N* HCl, 37°, 10 days) gave a brownish unidentifiable spot on a paper chromatogram, together with three main spots of DNP-aspartic acid, DNP-aspartylalanine and DNP-aspartylalanyl-X (*3*). The chromatographic behaviour on paper (developed with benzyl alcohol-ethanol-phthalate buffer and toluene system) and the absorption spectrum (a broad maximum near 400 mμ), instead of a sharp peak at 355–360 mμ) of the brown spot agreed perfectly with those for the acid decomposition product of mono-DNP-cystine residue in DNP-chymotrypsinogen reported by Bettelheim (*5*).

When the DNP-serum albumin was previously treated with performic acid by the method of Schram (*6*), the brown spot disappeared and a DNP-amino acid was newly detected in the hydrolysate, which was identified as DNP-cysteic acid by paper chromatography (*n*-butanol saturated with 0.5 per cent ammonia aq., Rf=0; phenol-water (4:1), Rf=0.50; *n*-butanol-acetic acid-water (4:1:3), Rf=0.35). Fig. 1 shows the amount of DNP-cysteic and -aspartic acids in the various hydrolysate of the oxidized DNP-protein, which were estimated in the following manner. After previous fractionation of the hydrolysate through columns of Amberlite IR-112 (Na^+ form) and talc, the non-basic DNP derivatives were chromatographed on a filter paper and the eluent of each spot with water was submitted to photometric determination at 350 mμ and 360 mμ.

The application of the usual PTC method (*3*, *7*) on the performic acid-treated serum albumin (*6*, *8*) gave phenylthiohydantoin (PTH)

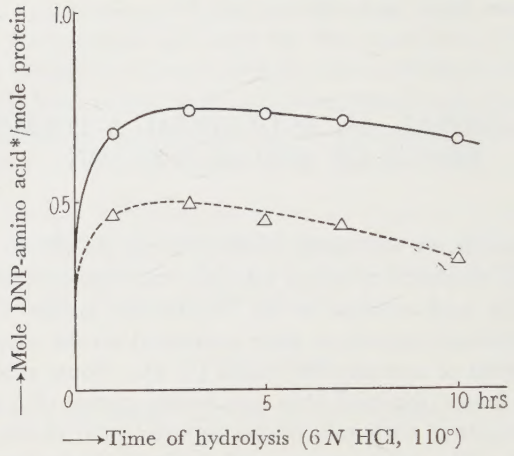


FIG. 1. The amount of DNP-cysteic (△) and -aspartic (○) acids in the various hydrolysate of the oxidized DNP-serum albumin.

* The decomposition of DNP-amino acid during the hydrolysis and the yield of oxidation of DNP-cystine to DNP-cysteic acid (*6*, *8*) were not corrected.

of aspartic and cysteic acids, which were identified by paper chromatography. The alcoholic extract of the cyclized reaction mixture (*7*) was developed with *n*-butanol saturated with *M*/10 citrate buffer (pH= 4.2) and colored with iodine-azide reaction (PTH-Asp Rf=0.85, PTH-$CySO_3H$, Rf=0.30).

The above-mentioned results suggest that serum albumin possesses two *N*-terminal groups, aspartic acid and half cystine, the latter of which has not been detected by the usual DNP or PTC method on the unoxidized protein and that the results of Thompson on the *N*-terminal sequence (*3*) and of White on the *C*-terminal one (*4*) necessiate further investigation based upon that the protein has two peptide chains.

REFERENCES

(*1*) Van Vunakis, H., and Brand, E., Abstracts, 119th meeting, American Chemical Society, p. 28, (1951)
(*2*) Desnuelle, P., Rovery, M., and Fabre, C., *Compt. rend.*, **233**, 987 (1951)
(*3*) Thompson, E. O.P., *J. Biol. Chem.*, **208**, 565 (1954)
(*4*) White, W. F., Shields, J., and Robbins, K. C., *J. Am. Chem. Soc.*, **77**, 1267 (1955)

(*5*) Bettelheim, F. R., *J. Biol. Chem.*, **212**, 235 (1955)
(*6*) Schram, E., Moore, S., and Bigwood, E. J., *Biochem. J.*, **57**, 33 (1954)
(*7*) Levy, A. L., and Li, C. H., *J. Biol. Chem.*, **217**, 355 (1955)
(*8*) Kimmel, J. R., Thompson, E.O.P., and Smith, E. I., *J. Biol. Chem.*, **217**, 151 (1955)

Department of Chemistry
Faculty of Science
University of Tokyo
KOITI TITANI
HIROSHI YOSHIKAWA
Department of Chemistry
Faculty of Science
Tokyo Metropolitan University
KAZUO SATAKE

(Received for publication, June 5, 1956)